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PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 172, 139–152 (1983)

The Function of Vitamin A: Current Concepts[1] (41537)

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Visual function of vitamin A. Somatic functions of vitamin A: a. differentiation. b. growth. *Reproduction. Membrane phenomena. Conclusions. Addendum.*

The function of vitamin A has remained an enigma even though this vitamin was one of the earliest to be discovered (1). The knowledge that has accumulated from more than half a century of investigations points to a multitude of physiological and biochemical changes that take place when the mammalian body has insufficient vitamin A. At this time it has become evident that at least three different physiological functions are dependent on proper vitamin A nutrition:

(1) Somatic function or systemic function—this encompasses growth and differentiation, and for vitamin A is best understood in terms of development and differentiation of epithelial structures and bone (2–6). Forms of vitamin A capable of supporting somatic functions include retinol, retinyl esters, retinal and retinoic acid.

(2) Reproduction—vitamin A is essential for spermatogenesis, oogenesis, placental development, and fetal and embryonic growth. While retinol, retinyl esters, and retinal are active for these functions, retinoic acid is *not* able to support all aspects of mammalian reproduction (2, 3, 7).

(3) Visual process—it is firmly established that vitamin A is required for vision in the dark, possibly also for color perception. The active form of vitamin A for this function is the aldehyde, retinal (8), derived from retinyl esters and retinol. Retinoic acid is inactive (9).

Some of the most significant developments in the area of vitamin A function and metabolism are outlined in this introductory section.

Utilization of vitamin A in the body is a tightly regulated process; there are several sites of control:

(1) Transport of vitamin A from its storage site in liver to tissues is in the form of retinol bound to its specific carrier protein, retinol-binding protein (10, 11). Retinoic acid is apparently transported by serum albumin (12).

(2) There is control at the level of entry into cells: it has been shown that a specific cell surface binding protein accepts the vitamin from its carrier protein and then transfers it across the membrane into the cell (13, 14).

(3) At the cellular level vitamin A appears to be bound to a cellular receptor protein: cytosolic binding proteins have been found that specifically bind retinol, retinal and retinoic acid (15–18), and there is evidence of nuclear receptors specific for retinol and retinoic acid (15, 18–23).

(4) Computer modeling studies suggest that plasma retinol cycles repeatedly in a "futile cycling" process that interfaces target issues, circulation, and body pools (M. H. Green, personal communication).

The question now arises: what exactly does vitamin A do at the cellular and molecular level? Although a great deal of effort has been expended in many laboratories to answer this question, the function of this important nutrient is still unknown. At the present time there are three working models offered to explain the mode of action of vitamin A.

One is based on the mode of action of vitamin A in vision, where, in the form of 11-*cis*-retinal, it functions as the prosthetic group on the visual protein, opsin, in the rhodopsin cycle ((8, 9), reviewed in (5, 6)). This molec-

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ular mechanism of action for vitamin A, however, so far has not been found applicable to other target sites of vitamin A function.

To explain the mode of action of vitamin A in reproductive and general somatic functions, such as growth and differentiation, there are currently two models under active investigation:

(1) Vitamin A acts in a coenzyme fashion as a sugar carrier for glycoprotein biosynthesis. Work is in progress in several laboratories to seek substantiating evidence for this hypothesis (5, 6, 24).

(2) Vitamin A functions via a specific receptor protein (16, 17). Since cellular binders for all active forms of vitamin A have been found (16, 17, 25, 26), this working model has received wide acceptance.

Functions of vitamin A must be intimately linked to its metabolism. Simultaneously with research ongoing in the area of vitamin A function, there has been a great deal of stimulating work in the area of vitamin A metabolism. Of interest has been the work of Wolf and De Luca, who have focused on retinyl phosphate as the metabolically active form of retinol. Retinyl phosphate has been detected in several tissues and is biologically (growth response) as active as retinol (27, 28). Retinyl phosphate combines with monosaccharides such as mannose and can transfer this sugar to a protein (24). The physiological significance of retinyl phosphate and the sugar–lipid intermediate formed remains to be established.

Extensive research has developed around the concept that retinoic acid or a metabolite of it might be the "active form" of vitamin A for its somatic function. This concept continues to be viable because reductive metabolism (to retinol, retinal, or retinyl esters) does not appear to occur *in vivo*. Storage forms (retinyl esters) are not generated and the metabolism of retinoic acid is rapid. Metabolism of retinoic acid is still not fully elucidated and thus metabolites remain yet to be identified and tested for vitamin A activity.

Several important developments have significantly contributed to the understanding of metabolism of retinoic acid:

(1) The realization that retinoic acid exists in many tissues and is a physiological metabolite of all known vitamin A-active compounds (29–32). This knowledge confirmed what was previously only speculation concerning the important role of retinoic acid as a vitamin A-active compound.

(2) The discovery that vitamin A compounds have anticarcinogenic potential and that among natural vitamin A compounds retinoic acid is the most promising antitumor agent (33–35). This finding has generated a tremendous interest within the medical field and has expanded our understanding of vitamin A in nutrition and metabolism.

(3) Geometric isomers of retinoic acid occur *in vivo* in certain target organs and may have physiological functions (36, 37).

(4) The development of new chromatographic and isolation technology for the separation of organic compounds. Methods such as liquid-gel partition chromatography (30, 38), improved isolation methods (36, 39), and especially high-pressure liquid chromatography [HPLC] (40–42) have practically revolutionized the field of vitamin A metabolism allowing separation of vitamin A compounds from each other and their isomers. Unfortunately, much of the earlier work on vitamin A compounds must now be viewed with some reservation because earlier methods permitted the destruction of the very labile vitamin A compounds.

It has now been possible to characterize several tissue metabolites of retinoic acid. One such metabolite is 5,6-epoxyretinoic acid (43), previously thought to be a highly active vitamin A compound (44, 45), but now proven to be almost totally inactive (46). Other recently identified metabolites of retinoic acid include 4-oxoretinoic acid and 4-hydroxyretinoic acid (47, 48); they, too, are biologically inactive and represent early catabolic products of vitamin A metabolism.

Another newly characterized metabolite of retinoic acid is retinoyl glucuronide (49–51) originally isolated and studied by Olson and co-workers (52–54). Postulated to be primarily a biliary metabolite of retinoic acid, retinoyl glucuronide was found to be 30–100% as active as all-*trans*-retinoic acid (52) and has been thought to represent the major excretory product of retinoic acid metabolism (53). However, the recent demonstration of only very small amounts of all-*trans*-retinoyl gluc-

uronide in bile (49–51) suggests that glucuronidation is a minor metabolic pathway for retinoic acid elimination. The excretion of other polar metabolites of retinoic acid as glucuronides is, however, very probable.

Of particular interest is the report that retinoyl glucuronide is the *major intestinal metabolite* from administered physiological concentrations of retinoic acid to vitamin A-deficient, bile duct-cannulated rats (36). The authors suggest that glucuronidation of retinoic acid to form the mixed anhydride-ketal, may have a physiological function in tissues.

Another recent finding is that in the intestinal mucosa, a target tissue for vitamin A action, retinoic acid exists in an equilibrium mixture, 2:1, of its all-*trans* and 13-*cis* forms, respectively (36). Although both forms appear to be equally active in the somatic function (growth response) (55), it has not been established if each form is active on its own account or because of a conversion to the other isomer. It is possible that *in vivo isomerization* of all-*trans*-retinoic acid is *crucial* to the cellular function of retinoic acid. Thus, 13-*cis* isomerization may be the initial physiological event in the action of retinoic acid at the cellular level, followed by further metabolic reactions. This idea gains support from studies with vitamin A-deficient rats: in the small intestinal mucosa of retinoic acid-dosed, bile duct-cannulated rats a similar equilibrium exists between the 13-*cis* and all-*trans* retinoyl glucuronides (36). Also, the 13-*cis* isomer of 4-keto-retinoic acid, although inactive, has been found to be a metabolite of all-*trans*-retinoic acid in liver (48). Recently, several isomers of retinoic acid have been detected in varying amounts in a number of tissues after administration of all-*trans*-retinoic acid to vitamin A-sufficient rats (37). *Cis-trans* isomers of retinol and retinal are also present in livers of mammals, birds, and fish (56, 57), suggesting that they are naturally occurring forms of vitamin A. The importance of isomerization for a particular expression of vitamin A activity thus may not be limited only to the rhodopsin cycle in retina but may be of a more universal occurrence.

Progress in elucidation of vitamin A metabolism has not yet led to an understanding of the function of vitamin A. Presently the function of this vitamin can not be explained by a single unifying mechanism involving a common metabolic intermediate. Equally active forms of vitamin A, such as retinol, retinal, and retinoic acid, may in fact each act by divergent mechanisms and may possibly even affect different functions in specific target tissues.

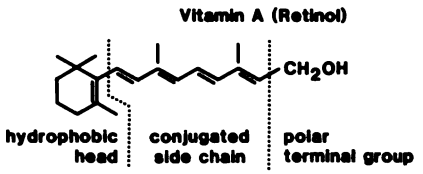

FIG. 1. Structure of vitamin A.

A brief examination of the vitamin A molecule (Fig. 1) reveals three important features: (1) a hydrophobic head: the β-ionone ring; (2) a conjugated isoprenoid side chain: this conjugated double bond system lends itself to isomerization (by light, or enzyme, or heat) and thus allows for a change in shape of the molecule; (3) a polar terminal group that can be enzymatically or chemically modified to become an ester as in retinyl palmitate or an aldehyde (-CHO) as in retinal or be oxidized to a very polar carboxylic (-COOH) group as in retinoic acid.

It is clear that a molecule with such diverse chemical properties of its components may readily enter into several different biochemical reactions, the choice of reaction type governed by endogenous and exogenous influences in the biological environment surrounding it.

Our discussion to follow is primarily directed at an examination of the current understanding of physiological function(s) of vitamin A in light of present and past concepts of possible mechanism(s) of action.

Visual Function of Vitamin A. Vitamin A, because of the diversity of its molecular components, can serve in various different biochemical functions at the molecular level. It is obvious that in the course of evolution advanced organisms have utilized this unique molecule in several biological systems.

The best example is the function of vitamin A aldehyde, retinal, in vision. A change in shape of the molecule, such as that resulting from isomerization of all-*trans*-retinal to 11-*cis* form, allows this molecule to fit the surface of a specific glycoprotein, opsin, located in the

rods of the retina, where it is bound to protein in an imine linkage (Schiff base). Here retinal acts as a specific chromophore that responds to white light by isomerization to all-*trans* form, releasing retinal from the protein and thus causing a change in charge distribution on the protein and initiating events that lead to neurotransmission. This process was discovered by Wald (8) and has been intensively studied and reviewed (5, 6).

The unique properties of vitamin A are also thought to account for color perception. In the *cones* of the retina, 11-*cis*-retinal fits into three specific cone opsins, each of which provides a different environment in terms of distribution of negative charges along the length of the molecule and enables retinal to absorb at visible (longer) wavelengths (bathochrome shift). When a wavelength characteristic of a certain color (e.g., red) enters the eye, the 11-*cis* retinal that is bound to opsin in the "red" cones can absorb this light and is isomerized to its all-*trans* form. This change in shape of the molecule dissociates the chemical bonds between retinal and the protein, causes a redistribution of charges and a change in current, that finally results in a signal to brain, perceived as red color.

This experimental model for color perception, described above, has been recently proposed by Honig *et al.* (58, 59) and is expected to be applicable to all color-perceiving visual systems.

Much of the understanding of the role of retinal as chromophore stems from studies on the purple membrane in *Halobacterium halobium* (60–63). This membrane contains the protein bacteriorhodopsin with all-*trans*- and 13-*cis*-retinal as its chromophore. Since bacteriorhodopsin is a small molecule (in contrast to the large protein molecules in rods and cones of the vertebrate eye), it has been possible to progress much more rapidly in understanding the functioning of the protein–retinal complex, the chemistry of which is similar to that of the visual pigment, rhodopsin. In the bacterial membrane system the energy of light, absorbed by the retinal chromophore on the bacteriorhodopsin, is converted into an electrochemical proton gradient across the membrane, providing a source of energy for this unusual salt-adapted microorganism.

Thus, molecular functions of several retin pigments have been clearly established. Ev as bacteriorhodopsin is a light-driven prot pump (61–63), another retinal pigment, hal rhodopsin, has been found to function as light-driven sodium ion pump (64). Finall in higher organisms, rhodopsin in the eye e ables them to utilize the visible region of t spectrum and transform a light stimulus in an electrical signal that causes neurotrar mission and perception of dim light and, p sibly, also perception of color.

The specific protein–retinal interactio that enable vitamin A to perform light-a sorbing functions are dependent not only the photosensitivity of retinal because of co jugated double-bond system, but also on h drophobic properties of the β-ionone ring the molecule and on the reactivity of the dehyde group on the side chain terminal; parts of the molecule are essential for this bi chemical event to be accomplished.

The Somatic Functions of Vitamin A. *Differentiation.* It is universally accepted th vitamin A is required for growth and diff entiation. These functions, however, are i separable processes in higher organisms, i volving complex functioning of many tissu and cells. Indeed, the earliest symptom of tamin A deficiency—a loss of appetite and ensuing decrease of growth, is followed multiple changes in tissue morphology, most involving changes in epithelial tissue diff entiation. All this inevitably points to an i volvement of vitamin A in some very fu damental process.

A general observation of the effect of tamin A upon differentiation is that the tamin most certainly exerts its action up bipotential cells: cells that have retained th differentiating ability in the growing and m ture animal and can differentiate in more th one direction. Vitamin A deficiency effects the cellular level are most readily seen in thc differentiating tissues that have a rapid tu over rate, such as epithelia of oral cavity, spiratory tract, gastrointestinal tract, urina tract, and ducts of various secretory glands 3, 6, 65). Similarly, effects of vitamin A embryonic development (66–68), develc ment of bone and teeth (69, 70), maintenan of spermatogenesis (7, 71), and limb reg eration (72–75) have also been explained

an effect upon differentiation. Early (76) as well as recent (reviewed in (25)) studies with cell cultures have substantiated the involvement of vitamin A in cellular differentiation.

Regulation of differentiation of eukaryotic cells is still one of the major unsolved problems in biology. Programming of cell function has multiple control mechanisms that are indirectly influenced by environmental factors. It has become increasingly clear that vitamin A nutriture may be one such factor involved in regulation of differentiation of certain tissues.

It is well established that lowered immunoresponsiveness is a sign of vitamin A deficiency (reviewed in (6, 77, 78)) and that an increased level of dietary vitamin A provides protection against infection (reviewed in (77)). Certain xenobiotics, such as TCDD (tetrachlorodiphenyldioxane) (79, 80), ethanol (81), phenobarbital (82, 83), PBB (polybrominated biphenyls), and PCB (polychlorinated biphenyls) (84–86) increase vitamin A turnover from liver stores. Other stress factors, such as infection, accentuate vitamin A utilization and decrease assimilation ((87), reviewed in (88)). It is clear that optimal vitamin A stores and intake have a protective effect against adverse enviromental factors that could lead to misguided gene expression and altered cell phenotype. A case in point is the accumulating epidemiological evidence that adequate vitamin A nutriture has a significant protective effect against lung cancer in smokers (89, 90).

Differentiation is almost certainly based upon gene expression. Many biochemical studies suggest that vitamin A affects nuclear events ((91–98), reviewed in (6)), but the exact molecular site of its action has not been identified.

The mechanism of action of steroid hormone-induced differentiation has been successfully explored by the receptor model (99, 100); this model proposes that specific proteins facilitate interactions with nucleus. The model is also being applied to study the role of vitamin A in differentiation. This approach to elucidation of vitamin A function at the molecular level, pioneered by Chytil and Ong (16, 17, 101), has gained wide acceptance because cytosolic and nuclear binding proteins for vitamin A-active compounds have been found in many tissues (16–23) and their concentrations appear to be regulated by vitamin A status (101).

Additional support that vitamin A modifies genomic expression comes from the recent demonstration of nuclear binding of retinol in liver (18, 23) and the observation that retinol regulates the concentration of poly(A) RNA (mRNA) in several target tissues (97, 101). In epidermal cells it has been possible to correlate alterations in mRNA concentration with appearance of specific keratins (97, 98). The exact nuclear sites of these events remain to be identified.

Cellular binding proteins for vitamin A-active compounds have also been detected in some transformed cells ((16) reviewed in (25)), suggesting that a vitamin A–receptor complex is involved in expression of the anticarcinogenic properties of this vitamin. Presence of cellular binding proteins, however, does not always correlate with retinoid-induced effects on proliferation or differentiation.

Although the receptor model is an excellent tool for investigating the specific site of action of vitamin A at the molecular level, this mechanism may not account for all vitamin A functions. A correlation between the presence of cellular vitamin A binding proteins and gene regulation is not of universal occurrence. An example is the abundance in the retina of "receptors" for almost every known vitamin A-active compound (26, 103), while retina is a tissue where the function of vitamin A in the visual cycle is known *not* to involve gene expression. Thus the physiological function(s) of these cellular binding proteins is not clear. There is recent evidence that in retina the proteins may function as inter- and intracellular carriers for the various forms of vitamin A (26, 102, 103). It is also possible that the proteins may be enzymes for the interconversion of the vitamin A compounds or they may be needed to stabilize the labile vitamin A compounds or to decrease the toxicity of free retinol and retinoic acid (104).

An argument against the receptor model has been raised in view of the data that there are vitamin A target tissues without detectable vitamin A-binding proteins as well as that there are nontarget tissues containing binding proteins (16, 17). It is likely, however, that these discrepancies exist because of our present incomplete knowledge of vitamin A target

tissues and the inability to detect small amounts of vitamin A binding proteins. The availability and application of sensitive immunochemical (105, 106) and radioimmunoassay (103, 107, 108) techniques may abrogate these differences.

The striking morphological changes in certain epithelial tissues that are the result of alterations in vitamin A nutrition (3, 5, 6, 65), are often accompanied by altered mucus secretion. Thus, a role of vitamin A in glycoprotein metabolism has been suggested to explain its effect on differentiation (5, 6, 24). The ability of vitamin A to alter glycoprotein biosynthesis has been documented in many *in vivo* and *in vitro* systems (5, 6, 24). Wolf and De Luca propose a *coenzyme* role for vitamin A, where retinol, as retinyl–phosphate–sugar complex, functions in membranes in post-translational transfer of monosaccharides to an acceptor protein, this resulting in synthesis of a specific glycoprotein that may affect cellular differentiation (5, 6, 24).

At the present time it has not been possible to demonstrate the existence of a protein that specifically accepts a sugar moiety from vitamin A. Furthermore, the model is not applicable to retinoic acid, even though this vitamin A-active compound stimulates glycoprotein synthesis and fulfills the somatic functions of vitamin A. Also lacking is a working hypothesis as to how a change in the biosynthesis of a vitamin A-dependent glycoprotein might result in modulation of cellular phenotype.

The demonstration that retinoic acid can regulate release of the glycoprotein, fibronectin, in an enucleated cell (109) indicates that *cytoplasmic* function may be another molecular mechanism for vitamin A.

b. Growth. Elucidation of somatic functions of vitamin A becomes even more complicated in the light of some important concepts of vitamin A action that have received very limited attention.

Pathological lesions associated with vitamin A deficiency in animals have been described in great detail (2, 3, 110), but it is not known what initial biochemical events are responsible for the eventual morphological changes. Physiological and biochemical changes have been observed in young animals when their dietary vitamin A is withheld even though they still have adequate amounts of vitamin A stored in their livers, and therefore have an endogenous supply of vitamin A that is supposedly "available" for release to needy tissues. The outward appearance of such animals, their growth rate, and their serum vitamin A give no indication of an ensuing vitamin A deficiency; the animals are in apparent good health. However, the development of tissues that have been examined was found to be restricted (77, 111). One explanation could be that the amount of vitamin A adequate to maintain body weight and general health may *not* be sufficient for optimal growth and development of *all* tissues, as is the case with testes (112). The mechanism(s) regulating the release of retinol–RBP from liver of *normal* animals is not understood. This process might partially depend on newly absorbed (dietary) vitamin A, mostly retinyl esters. In the absence of this exogenous stimulus, the rate of release of the retinol–RBP complex may be sufficient to keep the blood concentration of retinol normal, but *not* sufficient to satisfy the needs of rapidly proliferating tissues.

The principal cause of an impairment in growth in a nutritional insufficiency might be connected with alterations in protein metabolism (113). The initial biochemical effect thus could be either at the nuclear level or epigenetic; for example, some function of vitamin A may be concerned with maintenance of stability or specificity of complex proteins, particularly in membranes.

Recent studies have provided evidence that rapidly proliferating tissues are very sensitive to suboptimal vitamin A nutrition: DNA synthesis phase in the small intestinal crypt cells is prolonged (111), addition of new cells to growing organs is decreased (77), and DNA labeling index (114), cell mitoses (115), and DNA synthesis (116, 117) are decreased in several tissues of animals prior to manifestation of external deficiency symptoms. The lowering in DNA synthesis activity may be a conservation mechanism for vitamin A in the presence of a limited supply of this micronutrient. Under suboptimal nutritional conditions, cells cease to multiply and, to survive, may revert to a differentiated state that is better adapted to existing nutritional conditions.

oral effects in target tissues of decreased synthetic activity, followed by hyper-, might also be explained by a prolif- e dependence for vitamin A in rapidly ng target cells. Such cells would be ɔd in G_0 permitting other juxtaposed not dependent on vitamin A, to con- proliferating. Recent *in vivo* experi-, using vitamin A-depleted and -re- animals, suggest that both mechanisms ɔe operating simultaneously in tracheal ɛlium (114, 118, 119). Whether these ɛs in cellular behavior caused by vita- A insufficiency are phenotypic altera- of target cells or the "overgrowth" of nondependent cell types *in vivo*, cannot swered with our present knowledge.

dies with rats in germ-free environment 121) have been very useful in separating ıs components associated with vitamin ction: growth, differentiation, reproduc- vision. The germ-free experimental l illustrates clearly the vitamin A re- nent for reproduction, vision in the dark, pithelial tissue differentiation: such an- cannot reproduce or see in the dark; epithelia undergo keratinization; ac- lated keratinized debris obstructs vital ges to organs and is often the primary of death of the animal.

he germ-free environment rats can live ıaintain their body weight in the absence ımin A. The experimental model illus- a very important concept: if all stresses moved, animals can survive without vi- A. However, if demands are imposed the body, such as growth or need for tissue regeneration or turnover, the an- *must* have vitamin A to survive. This pt is also supported by the observation he requirement of vitamin A depends ɛ rate of growth (122–126, also reviewed). Similarly, bone lesions due to inade- vitamin A nutrition are observed only ung, growing animals (2–6) and are bly related to the need for this vitamin ɔliferation and differentiation of bone particularly osteoblasts and osteoclasts. te extensive studies with hypo- and hy- aminotic-A animals (2–6), regulation by in A of bone growth and development completely obscure.

production. The requirement of vitamin A for reproductive functions in higher animals has been known since 1922 (127). In studies with animals restricted to vitamin A-deficient diets and repleted with various active forms of vitamin A, Thompson *et al.* (7) established a dichotomy of vitamin A function: while retinoic acid maintained somatic epithelial function in testes, it did not maintain germinal epithelium or spermatocytogenesis. The reduced forms of vitamin A, retinol, or retinal, are required for these functions. In female rats, hormone production, oogenesis, fertilization, and implantation are supported equally well by retinoic acid, retinol, and retinal, but the reduced forms are required for placental and fetal development.

Recent studies, however, suggest that in addition to its general, somatic role in epithelial maintenance, retinoic acid *does* have a specific function in testes: it supports testosterone biosynthesis in Leydig (interstitial) cells (128). Both retinol and retinoic acid appear to be required for complete testicular function. It remains to be determined why retinoic acid alone *can* support spermatogenesis and oogenesis in birds (129).

The partial vitamin A activity of retinoic acid has been very useful in sorting out the various functions of vitamin A, particularly in separating the multiple somatic lesions (that often lead to complicating consequences) from the more specific effect of vitamin A on reproduction (and vision). At this time it is still not clear what biochemical systems are involved.

Wald and Dowling suggested (9) that retinol serves merely as a precursor to two active forms of vitamin A: retinal and retinoic acid. This idea is still a valid model for proposing a function for *retinal* in reproduction: (a.) retinal supports *all aspects* of reproduction (7); (b.) retinol–retinal interconversions occur in tissues ((30, 130) discussed in (6)). Cellular binding proteins for retinal have been detected in eye tissues (26, 102, 158); no data on other tissues are available. At this time one can not assume that retinal is functional only in vision. Present evidence supports the concept that reproduction in higher animals is an example of a specialized system that utilizes several available active forms of vitamin A, each for its unique potential.

The *mechanisms of action* of vitamin A

proposed for maintenance of growth and differentiation have also been suggested for the function of vitamin A in reproduction. Cellular binding proteins for both retinol and retinoic acid have been isolated from testes, ovary and uterus (16), oviduct (131) and mammary gland (132). Extensive studies with testicular retinol- and retinoic acid-specific binding proteins have strengthened the view that the action of vitamin A compounds in reproduction is mediated by specific intracellular carrier proteins and that vitamin A exerts its effect on reproduction at the gene level via a nuclear receptor.

Membrane Phenomena. Vitamin A, when added in excess to biological systems, acts on cellular membranes and glycocalyx. Membrane effects have shown a high degree of correlation with biological activity. As a result of these observations as well as the surface-active effect of vitamin A (see Fig. 1), a working hypothesis has been proposed by Lucy (133) that one function of this vitamin may be to physically alter lipoprotein membranes and thus to affect differentiation.

Effects of vitamin A on membrane structure and function have been studied extensively in biological and physical experimental systems. When high nonphysiological concentrations of vitamin A are employed, biological studies demonstrate that free retinol (not bound to its physiological carrier, RBP), labilizes membranes causing leakage of enzymes (134–136). Lucy (133) has suggested that physiological concentration of vitamin A might similarly regulate release of membrane-enclosed enzymes essential for normal metabolic processes and thus account for the varied functions influenced by vitamin A.

For explanation of the physiological function of vitamin A the membrane model has been discounted at this time, since there are several lines of evidence that indicate that the hypervitaminotic effects of vitamin A are *not related* to the physiological function of this vitamin. For example, α-retinol, an analog of retinol capable of producing the membrane effects, has very low growth-promoting activity (135). Also, an excessive amount of retinol, when added to cell culture in its physiologically circulating form (bound to RBP), did not cause the deleterious alterations in membrane structure and function that are associated with the action of free retinol (137). Furthermore, considering the very low concentration of vitamin A in membranes (except in the membrane discs of retinal rods), it is difficult to envision this compound in the role of a structural component.

Much of the available evidence, however, *does* point to *membranes* as the most likely biological structures for the site of action of vitamin A (138–144). Also, length of the vitamin A molecule is appropriate to span the lipid bilayer of some cellular membranes (145).

Although vitamin A is distributed among various soluble intracellular compartments, some of it is *always* associated with membranous structures (138, 139, 146, 147). Furthermore, in recent studies demonstrating the delivery of retinol via cellular receptor to nucleus, it was noted that free retinol was associated with the nuclear membrane (23).

Evidence is increasing that membranous structures play an essential part in many cellular events. Since vitamin A influences the *biosynthesis of glycoproteins*, characteristic components of membranes, it may affect cellular differentiation by a nonnuclear mechanism. Glycoproteins on the surface of mammalian cells are important in a variety of membrane-mediated functions, such as cell adhesion and communication. *In vitro* studies have demonstrated that these cellular interactions are affected by vitamin A ((140–143), reviewed in (25)). Evidence also suggests that changes in membrane glycosylation affect cell surface recognition mechanisms (148–150) and that glycosylation of membrane-associated proteins may be an integral part of cellular differentiation (151).

Recent morphogenesis studies indicate that vitamin A compounds exert striking systemic effects on the anatomical patterns of regenerating limbs in amphibians (72–75) and developing limb buds in chick and mouse (152–155). Maden (74) suggests that the effect of retinoids on cell surface glycoprotein glycosylation, on gap junction formation, and on secretion of matrix proteins may be responsible for alterations in cellular communication during limb pattern formation and thus may influence the determination and maintenance of tissue positional information.

The highly reactive nature of the molecular

es of vitamin A indicates that this com-
id could be involved in electron transfer.
' ((133), see also (6)) has suggested that
nin A may be involved in the *electron*
fer chain of the membrane-bound en-
s [e.g., Cyt P_{450}-mixed function oxi-
s] in microsomes. This possibility is
gthened by the observation in model sys-
that flavins catalyze the isomerization of
al (156).

ucidation of the molecular function
tinal as a photosensitive chromophore,
rfully demonstrates how the various parts
e vitamin A molecule can be utilized by
ell to accomplish a biological task. The
oprocess (photoisomerization) induces a
ormational change in membrane protein,
leading to an increased permeability of
nembrane to ions and to the consequent
ration of a charge. While not directly ap-
ble to the elucidation of other physio-
al functions of vitamin A, the retinal
mophore *does* provide us with an addi-
al working model: in the somatic cells vi-
n A-active compounds could be *enzy-
cally isomerized* and then utilized, in a
lar fashion, to perform some fundamen-
iochemical function.

is definitely premature to speculate
her some action of vitamin A at the gene
affects membrane function or whether
function of vitamin A at the membrane
alters gene expression.

nclusions. All available evidence indi-
that vitamin A has a very complex func-
in the body, involving cellular prolifer-
, differentiation, as well as specialized
tions, such as vision and reproduction.
e search for a link between a biochem-
reaction, initiated by vitamin A, and a
iological or morphological change, has
ressed in several directions. The follow-
models are being explored to study the
lation of molecular events by vitamin A:

) Isomerization of vitamin A to affect
ormation and charge of complex pro-
. Although this model has been useful to
date the function of vitamin A in vision,
r it has not been found applicable to so-
c functions of this vitamin. It is likely that
gher organisms an enzymatic isomeriza-
will link a critical biochemical event
to the physiological change elicited by
vitamin A.

(2) Regulation of nuclear events via specific vitamin A–cellular binding protein complexes. This model appears to explain the effect of vitamin A in certain target epithelia. However, specific alterations in proliferative or differentiative response do not always correlate with presence of vitamin A binding proteins.

(3) Modulation of glycoprotein biosynthesis by participation in sugar transfer reactions. The importance of glycoprotein processing in cellular control mechanisms makes this hypothesis attractive. Demonstration of a specific protein acceptor and identification of a retinoic acid intermediate would further substantiate this hypothesis.

(4) Membrane phenomena. It is possible that the ability to penetrate and modify biological membranes is the basis for some physiological activity of vitamin A such as stabilizing complex protein, i.e., membrane-bound enzymes.

(5) Electron transfer. The conjugated double-bond system of vitamin A molecule can provide an electron mobility that might be utilized for some systemic actions.

It is certain that a single unifying mechanism cannot explain the molecular action of vitamin A. Several active forms of this vitamin exist in the body: retinol, retinal, and retinoic acid. They may function each in a specific tissue or simultaneously in the same tissue, possibly each modulating cellular behavior by a different mode of action.

The significance of retinoic acid is still not clear. Is retinoic acid a partially active metabolite of vitamin A or *must* retinol and retinal be converted to retinoic acid for the vitamin A function in growth and maintenance of somatic epithelial tissues? So far, it is not known whether the biochemical event caused by retinoic acid is specific only for retinoic acid, because the reduced forms of vitamin A are as effective as retinoic acid and can be physiologically metabolized to retinoic acid. A specific function could be ascribed to retinoic acid with the demonstration of a differentiation product specific only for retinoic acid in a target tissue that responds to all forms of vitamin A.

Addendum. Vitamin A research has been characterized by a considerable progress in recent years. This is reflected not only in the vast number of publications in the area, but also in the availability of several excellent *recent* reviews. Particularly informative is the *comprehensive survey* and evaluation of the vitamin A field by Wolf (6). Other recent reviews dealing with *specific* areas include: an update of RBP research (11), an examination of natural and synthetic vitamin A compounds (collectively designated as retinoids) and their *in vivo* and *in vitro* effect on neoplasia (25); a review on the involvement of vitamin A in sugar transfer reactions in mammalian membranes (24); a discussion of cellular binding proteins for vitamin A compounds (16, 18); a general review of vitamin A, including nutritional information, and chemical and biological assay procedures (88); reviews on vitamin A and cancer (33–35); a review on vitamin A deficiency as a public health problem in many parts of the world (157) and a review of retinoids in ocular tissues (158).

1. McCollum EV, Davis M. The nature of dietary deficiencies of rice. J Biol Chem 23:181, 1915.
2. Moore T. Vitamin A. New York, Elsevier, 1957.
3. Wolbach SB. Effects of vitamin A deficiency and hypervitaminosis A in animals. In: Sebrell WH, Harris RS, eds. The Vitamins. New York, Academic Press, Vol I:pp106–137, 1954.
4. Barnicot NA, Datta SP. Vitamin A and bone. In: Bourne GH, ed. The Biochemistry and Physiology of Bone. New York, Academic Press, Vol II:pp197–229, 1972.
5. De Luca LM. Vitamin A. In: DeLuca HF, ed. Handbook of Lipid Research. The Fat Soluble Vitamins. New York, Plenum, Chap 1, pp1–67, 1978.
6. Wolf G. Vitamin A. In: Alfin-Slater RB, Kritchevsky D, eds. Human Nutrition. New York, Plenum, Vol 3B:pp97–203, 1980.
7. Thompson JN, Howell JMcC, Pitt GAJ. Vitamin A and reproduction in rats. Proc Roy Soc London 159:510–535, 1964.
8. Wald G. The visual function of vitamin A. In: Vitamins and Hormones. New York, Academic Press, Vol 18:pp417–430, 1960.
9. Dowling JE, Wald G. The biological function of vitamin A acid. Proc Nat Acad Sci USA 46:587–608, 1960.
10. Kanai M, Raz A, Goodman DeW. Retinol-binding protein: Transport protein for vitamin A in human plasma. J Clin Invest 47:2025–2944, 1968.
11. Rask L, Anundi H, Böhme J, Eriksson U, Frederiksson A, Nilsson SF, Ronne H, Vahlquist A, Peterson PA. The retinol-binding protein. Scand J Clin Lab Invest 40, Suppl 154, pp45–61, 1980.
12. Smith JE, Milch PO, Muto Y, Goodman DS. The plasma transport and metabolism of retinoic acid in the rat. Biochem J 132:821–827, 1973.
13. Heller J. Interactions of plasma retinol-binding protein with its receptor. Specific binding of bovine and human retinol-binding protein to pigment epithelium from bovine eyes. J Biol Chem 250:3613–3619, 1975.
14. Rask L, Peterson PA. In vitro uptake of vitamin A from retinol-binding plasma protein to mucosal epithelial cells from the monkey's small intestine. J Biol Chem 251:6360–6366, 1976.
15. Ong DE, Chytil F. Changes in levels of cellular retinol- and retinoic acid-binding proteins of liver and lung during perinatal development of rat. Proc Nat Acad Sci USA 73:3976–3978, 1976.
16. Chytil F, Ong DE. Cellular-binding proteins for compounds with vitamin A activity. In: Receptors in Hormone Action. New York, Academic Press, Vol II:pp573–591, 1978.
17. Chytil F, Ong DE. Cellular retinol- and retinoic acid-binding proteins in vitamin A action. Fed Proc 38:2510–2514, 1979.
18. Chytil F. Liver and cellular vitamin A binding proteins. Hepatology 2:282–287, 1982.
19. Sani BP, Donovan K. Localization of retinoic acid-binding protein in nuclei and the nuclear uptake of retinoic acid. Cancer Res 39:2492–2496, 1979.
20. Rao MRS, Prasad VR, Padmanaban G, Ganguly J. Isolation and characterization of binding proteins for retinol from the cytosol, nucleosol and chromatin of the oviduct magnum of laying hens. Biochem J 183:501–506, 1979.
21. Russell P, Wiggert B, Derr J, Albert D, Craft J, Chader G. Nuclear uptake of retinoids: Autoradiographic evidence in retinoblastoma cells in vitro. J Neurochem 34:1557–1560, 1980.
22. Takase S, Ong DE, Chytil F. Cellular retinol-binding protein allows specific interaction of retinol with nucleus in vitro. Proc Nat Acad Sci USA 76:2204–2208, 1979.
23. Liau G, Ong DE, Chytil F. Interaction of the retinol/cellular retinol-binding protein complex with isolated nuclei and nuclear components. J Cell Biol 91:63–68, 1981.
24. De Luca LM. The direct involvement of vitamin A in glycosyl transfer reactions in mammalian membranes. In: Vitamins and Hormones. New York, Academic Press, Vol 35:pp1–57, 1977.
25. Lotan R. Effects of vitamin A and its analogs (retinoids) on normal and neoplastic cells. Biochim Biophys Acta 605:33–91, 1980.
26. Saari JC, Bredberg L, Garwin G. Endogenous retinoids associated with three cellular retinoid-binding proteins from bovine retina. Fed Proc 41:861 (Abstract), 1982.

7. Rosso GC, De Luca L, Warren CD, Wolf G. Enzymatic synthesis of mannosyl retinyl phosphate from retinyl phosphate and GDP-mannose. J Lipid Res 16:235–243, 1975.
8. Frot-Coutaz JP, Silverman-Jones CS, De Luca LM. Isolation, characterization and biological activity of retinyl phosphate from hamster intestinal epithelium. J Lipid Res 17:220–230, 1976.
9. Emerick RJ, Zile M, DeLuca HF. Formation of retinoic acid from retinol in the rat. Biochem J 102:606–611, 1967.
0. Ito YL, Zile M, Ahrens H, DeLuca HF. Liquid-gel partition chromatography of vitamin A compounds; formation of retinoic acid from retinyl acetate in vivo. Lipid Res 15:517–524, 1974.
1. DeRuyter MG, Lambert WE, DeLeenheer AP. Retinoic acid: An endogenous compound of human blood. Unequivocal demonstration of endogenous retinoic acid in normal physiological conditions. Anal Biochem 98:402–409, 1979.
2. Shidoji Y, Hosoya N. Competitive protein-binding radioassay for retinoic acid. Anal Biochem. 104:457–463, 1980.
3. Bollag W. Retinoids and cancer. Cancer Chemother Pharmacol 3:207–215, 1979.
4. Newberne PM, Rogers AE. Vitamin A, Retinoids and Cancer. In: Newell GR, Ellison NM, eds. Nutrition and Cancer: Etiology and Treatment. New York, Raven Press, pp217–231, 1981.
5. Sporn MB, Newton DL. Retinoids and chemoprevention of cancer. In: Zedeck MS, Lipkin M, eds. Inhibition of Tumor Induction and Development. New York, Plenum, Vol 3:pp71–100, 1981.
6. Zile MH, Inhorn RC, DeLuca HF. Metabolism in vivo of all-trans retinoic acid. Biosynthesis of 13-cis-retinoic acid and all-trans- and 13-cis-retinoyl glucuronides in the intestinal mucosa of the rat. J Biol Chem 257:3544–3550, 1982.
7. Sundaresan PR, Bhat PV. Ion-pair high pressure liquid chromatography of cis-trans isomers of retinoic acid in tissues of vitamin A-sufficient rats. J Lipid Res 23:448–455, 1982.
8. Nystrom E, Sjovall J. Separation of lipids on methylated Sephadex. Anal Biochem 12:235–248, 1965.
9. Ito Y, Zile M, DeLuca HF, Ahrens HM. Metabolism of retinoic acid in vitamin A-deficient rats. Biochim Biophys Acta 369:338–350, 1974.
0. Frolik CA, Tavela TE, Sporn MB. Separation of the natural retinoids by high-pressure liquid chromatography. J Lipid Res 19:32–37, 1978.
1. McCormick AM, Napoli JL, DeLuca HF. High-pressure liquid chromatographic resolution of vitamin A compounds. Anal Biochem 86:25–33, 1978.
2. McKenzie RM, Hellwege DM, McGregor ML, Rockley NL, Riquetti PJ, Nelson EC. Separation and identification of geometric isomers of retinoic acid and methyl retinoate. J Chromatogr 155:379–387, 1978.
3. McCormick AM, Napoli JL, Yoshizawa S, DeLuca HF. 5,6-Epoxyretinoic acid as a physiological metabolite of retinoic acid in the rat. Biochem J 186:475–481, 1980.
4. John KV, Lakshmanan MR, Cama HR. Preparation, properties, and metabolism of 5,6-monoepoxyretinoic acid. Biochem J 103:539–543, 1967.
5. Krishna Mallia A, John J, John KV, Lakshmanan MR, Jungalwala FB, Cama HR. Biological activity of epoxides of vitamin A. Indian J Biochem 7:102–103, 1970.
6. Zile MH, Inhorn RC, DeLuca HF. The biological activity of 5,6-epoxyretinoic acid. J Nutr 110:2225–2230, 1980.
7. Frolik CA, Roberts AB, Tavela TE, Roller PP, Newton DL. Isolation and identification of 4-hydroxy- and 4-oxoretinoic acid. In vitro metabolites of all-trans-retinoic acid in hamster trachea and liver. Biochemistry 18:2092–2097, 1979.
8. Frolik CA, Roller PO, Roberts AB, Sporn MB. In vitro and in vivo metabolism of all-trans- and 13-cis-retinoic acid in hamsters. J Biol Chem 225:8057–8062, 1980.
9. Zile MH, Schnoes HK, DeLuca HF. Characterization of retinoyl-β-glucuronide as a minor metabolite of retinoic acid in bile. Proc Nat Acad Sci USA 77:3230–3233, 1980.
0. Swanson BN, Frolik CA, Zaharevitz DW, Roller PP, Sporn MB. Dose-dependent kinetics of all-trans retinoic acid in rats. Biochem Pharmacol 30:107–113, 1981.
1. Zile MH, Inhorn RC, DeLuca HF. Metabolites of all-trans-retinoic acid in bile: Identification of all-trans- and 13-cis-retinoyl glucuronides. J Biol Chem 257:3537–3543, 1982.
2. Nath K, Olson JA. Natural occurrence and biological activity of vitamin A derivatives in rat bile. J Nutr 93:461–469, 1967.
3. Lippel K, Olson JA. Origin of some derivatives of retinoic acid found in bile. J Lipid Res 9:580–586, 1968.
4. Olson JA. Some aspects of vitamin A metabolism. In: Harris RS, Wool IG, Loraine JA, eds. Vitamins and Hormones. New York, Academic Press, Vol 26:pp1–63, 1968.
5. Zile M, Emerick RJ, DeLuca HF. Identification of 13-cis retinoic acid in tissue extracts and its biological activity in rats. Biochim Biophys Acta 141:639–641, 1967.
6. Braekkan OR, Myklestad H, Njaa LR, Utne F. Vitamin A isomers in the livers of rats and chicks. Nature (London) 186:312, 1960.
7. Stainer DW, Murray TK, Campbell JA. Isomerization of 11-cis vitamin A in vivo. Canad J Biochem Physiol 38:1219–1222, 1960.
8. Arnaboldi M, Motto MG, Tsujimoto K, Balogh-Nair V, Nakanishi K. Hydroretinals and hydrorhodopsins. J Amer Chem Soc 101:7082–7084, 1979.
9. Honig B, Dinur U, Nakanishi K, Balogh-Nair V, Gawinowicz MA, Arnaboldi M, Motto MG. An ex-

ternal point-charge model for wavelength regulation in visual pigments. J Amer Chem Soc **101**:7084–7086, 1979.

60. Oesterhelt D, Stoeckenius W. Rhodopsin-like protein from the purple membrane of *Halobacterium halobium*. Nature New Biol **233**:149–152, 1971.
61. Oesterhelt D. Bacteriorhodopsin as a light-driven proton pump. In: Azzone GS, Klingenberg M, Siliprandi N, eds. Membrane Proteins in Transport and Phosphorylation. Amsterdam, Elsevier, pp78–84, 1974.
62. Schreckenbach T, Walckhoff B, Oesterhelt D. Studies on the retinal-protein interaction in Bacteriorhodopsin. Eur J Biochem **76**:499–511, 1977.
63. Spudich JL, Stoeckenius W. Light-regulated retinal-dependent reversible phosphorylation of *Halobacterium* proteins. J Biol Chem **255**:5501–5503, 1980.
64. Lanyi J. Halorhodopsin—A second retinal pigment in *Halobacterium halobium*. Trends Biochem Sci **6**:60–62, Feb 1981.
65. Parnell JP, Sherman BS. Effect of vitamin A on keratinization in the vitamin A-deficient rat. In: Butcher EO, Sognnaes RF, eds. Fundamentals of Keratinization. Washington, DC, American Association for the Advancement of Science, pp114–131, 1962.
66. Langman J, Welsch GW. Excess vitamin A and development of the cerebral cortex. J Comp Neurol **131**:15, 1967.
67. Kochar DM. Studies on vitamin A-induced teratogenesis: Effects on embryonic mesenchyme and epithelium, and on incorporation of H^3-thymidine. Teratology **1**:299, 1968.
68. Thompson JN. Vitamin A in development of the embryo. Amer J Clin Nutr **22**:1063, 1969.
69. Mellanby EA. A Story of Nutritional Research. Baltimore, Williams & Wilkins, p74, 1950.
70. Hayes KC, Cousins RJ. Vitamin A deficiency and bone growth. I. Altered drift patterns. Calcif Tissue Res **6**:120–132, 1970.
71. Howell JMcC, Thompson JN, Pitt GAJ. Histology of the lesions produced in the reproductive tract of animals fed a diet deficient in vitamin A alcohol but containing vitamin A acid. I. The male rat. J Reprod Fertil **5**:159, 1963.
72. Niazi IA, Saxena S. Relationship between inhibiting influence of vitamin A and developmental stage of regenerating tail in toad tadpoles (*Bufo andersonii*). Indian J Exp Biol **17**:866–868, 1979.
73. Niazi IA, Jangir OP, Sharma KK. Forelimb regeneration at wrist level in adults of Skipper frog *Rana cyanophlyctis* (Schneider) and its improvement by vitamin A treatment. Indian J Exp Biol **17**:435–437, 1979.
74. Maden M. Vitamin A and pattern formation in the regenerating limb. Nature (London) **295**:672–675, 1982.
75. Cooke J. Vitamin A, limb patterns and the search for the positional code. Nature (London) **296**:603–605, 1982.
76. Fell HB, Rinaldini LM. The effects of vitamins A and C on cells and tissues in culture. In: Willman EN, ed. Cells and Tissues in Culture; Methods, Biology and Physiology. New York, Academic Press, Vol I:pp659–699, 1965.
77. Zile MH, Bunge EC, DeLuca HF. On the physiological basis of vitamin A-stimulated growth. J Nutr **109**:1787–1796, 1979.
78. Hof H, Wirsing CH. Anti-infective properties of vitamin A. Z Ernahrungswiss **18**:221–232, 1979.
79. Thunberg T, Ahlborg UG, Johnsson H. Vitamin A (retinol) status in the rat after a single oral dose of 2,3,7,8-tetrachlorodibenzo-p-dioxin. Arch Toxicol **42**:265–274, 1979.
80. Thunberg T, Ahlborg UG, Hakansson H, Krantz C, Monier M. Effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin on the hepatic storage of retinol in rats with different dietary supplies of vitamin A (retinol). Arch Toxicol **45**:273–285, 1980.
81. Sato M, Lieber CS. Increased metabolism of retinoic acid after chronic ethanol consumption in rat liver microsomes. Arch Biochem Biophys **213**:557–564, 1982.
82. Tuchweber B, Bhagwan DG, Salas M. Microsomal enzyme inducers and hypervitaminosis A in rats. Arch Pathol Lab Med **100**:100–105, 1976.
83. Backes WL, Krause RF, Canaday WJ. Observations relating phenobarbital induction of cytochrome P-450 to the obligatory destruction or release of vitamin A. Nutr Rep Int **19**:9–14, 1979.
84. Innami S, Nakamura A, Miyazaki M, Nagayama S, Nishide E. Further studies on the reduction of vitamin A content in the livers of rats given polychlorinated biphenyls. J Nutr Sci Vitaminol **22**:409–418, 1976.
85. Kato N, Kato M, Kimura T, Yoshida A. Effect of dietary addition of PCB, DDT or BHT and dietary protein on vitamin A and cholesterol metabolism. Nutr Rep Int **4**:437–445, 1978.
86. Darjono DVM. Vitamin A Status, Polybrominated Biphenyl Toxicosis and Common Bile Duct Hyperplasia. Ph.D. thesis, Michigan State University, 1982.
87. LeFrancois P, Lamblin G, Carles C, Maire G. Variations of some plasma parameters (albumin, prealbumin, retinol binding protein) and urinary parameters during measles in Senegalese children. Ann Nutr Aliment **33**:417–427, 1979.
88. Olson J. Vitamin A. In: Machlin L, ed. Handbook on Vitamins. New York, Marcel Dekker, Chap 1, in press, 1982.
89. Bjelke E. Dietary vitamin A and human lung cancer. Int J Cancer **15**:561–565, 1975.
90. Wald N, Idle M, Boreham J, Bailey A. Low serum-

vitamin-A and subsequent risk of cancer. Preliminary results of a prospective study. Lancet, 813–815, 1980.
1. Zachman RD. The stimulation of RNA synthesis in vivo and in vitro by retinol (vitamin A) in the intestine of vitamin A-deficient rats. Life Sci 6:2207–2213, 1967.
2. De Luca L, Little EP, Wolf G. Vitamin A and protein synthesis by rat intestinal mucosa. J Biol Chem 244:701–708, 1969.
3. Johnson BC, Kennedy M, Chiba N. Vitamin A and nuclear RNA synthesis. Amer J Clin Nutr 22:1048–1058, 1969.
4. Zile M, DeLuca HF. Vitamin A and ribonucleic acid synthesis in rat intestine. Arch Biochem Biophys 140:210–214, 1970.
5. De Luca L, Wolf G. Mechanism of action of vitamin A in differentiation of mucus-secreting epithelia. J Agr Food Chem 20:474–476, 1972.
6. Blalock JE, Gifford GE. Retinoic acid (vitamin A acid) induced transcriptional control of interferon production. Proc Nat Acad Sci USA 74:5382–5386, 1977.
7. Fuchs E, Green H. Multiple keratins of cultured human epidermal cells are translated from different mRNA molecules. Cell 17:573–582, 1979.
8. Fuchs E, Green H. Regulation of terminal differentiation of cultured human keratinocytes by vitamin A. Cell 25:617–625, 1981.
9. Jensen EV, DeSombre ER. Estrogen–receptor interaction. Science 182:126–134, 1973.
10. O'Malley BW, Means AR. Female steroid hormones and target cell nuclei. Science 183:610–620, 1974.
11. Chytil F, Omori M, Laiu G, Ong DE. Functions of vitamin A in differentiation. Fed Proc 41:861, 1982 (Abstract).
12. Bridges CDB. Function of vitamin A in vision. Fed Proc 41:860, 1982 (Abstract).
13. Adachi N, Smith JE, Sklan D, Goodman DS. Radioimmunoassay studies of the tissue distribution and subcellular localization of cellular retinol-binding protein in rat. J Biol Chem 256:9471–9476, 1981.
14. Ross AC, Takahashi YI, Goodman DS. The binding protein for retinol from rat testes cytosol. Isolation and partial characterization. J Biol Chem 253:6591–6598, 1978.
15. Ong DE, Chytil F. Immunochemical comparisons of vitamin A binding proteins of rat. J Biol Chem 254:8733–8735, 1979.
16. Ong DE, Chytil F. Immunochemical studies on cellular vitamin A binding proteins. Ann NY Acad Sci 359:415–417, 1981.
17. Conrad DH, Wirtz GH. Radioimmunoassay for vitamin A. Fed Proc 31:685, 1972 (Abstract).
18. Westfall SS, Wirtz GH. Vitamin A antibodies: Application to radioimmunoassay. Experientia 36:1351–1352, 1980.
109. Bolmer SD, Wolf G. An extra-nuclear function of retinoic acid. Fed Proc 41:740, 1982 (Abstract).
110. Hayes KC. On the pathophysiology of vitamin A deficiency. Nutr Rev 29:3–6, 1971.
111. Zile MH, Bunge EC, DeLuca HF. Effect of vitamin A deficiency on intestinal cell proliferation in the rat. J Nutr 107:552–560, 1977.
112. Bieri JG, Mason KE, Prival EL. Testis requirement for vitamin A in the rat and the effect of essential fatty acids. Int J Vitam Res 38:312–319, 1968.
113. Miller SA. Nutrition in the neonatal development of protein metabolism. Fed Proc 29:1497–1502, 1970.
114. Zile MH, Bunge EC, DeLuca HF. DNA labeling of rat epithelial tissues in vitamin A deficiency. J Nutr 111:777–788, 1981.
115. Sherman BS. The effect of vitamin A on epithelial mitosis in vitro and in vivo. J Invest Dermatol 37:469–480, 1961.
116. Takahashi YI, Smith JE, Winick M, Goodman DS. Vitamin A deficiency and fetal growth and development in the rat. J Nutr 105:1299–1310, 1975.
117. Jayaram M, Sarada K, Ganguly J. Effect of depletion of vitamin A, followed by supplementation with retinyl acetate or retinoic acid, on regeneration of rat liver. Biochem J 146:501–504, 1975.
118. Chopra DP. Induction and retinoid reversal of squamous metaplasia in organ cultures of vitamin A-deficient hamster trachea: Cytokinetic and ultrastructural changes. AACR Abstract 343, 1982.
119. McDowell EM, Keenan KP. Restoration of mucociliary epithelium in tracheas of vitamin A-deficient hamsters. Fed Proc 41:622, 1982 (Abstract).
120. Bieri JG, McDaniel EG, Rogers WE Jr. Survival of germ free rats without vitamin A. Science 163:574–575, 1969.
121. Rogers WE Jr, Bieri JG, McDaniel EG. Vitamin A deficiency in the germ-free state. Fed Proc 30:1773–1778, 1971.
122. Gilbert HR, Hart GH. Minimum vitamin A requirements with particular reference to cattle. J Nutr 10:409–427, 1935.
123. Paul HE, Paul MF. The relation of vitamin A intake to length of life, growth, tooth structure and eye condition. J Nutr 31:67–78, 1946.
124. Brown EF, Morgan AF. The effect of vitamin A deficiency upon the nitrogen metabolism of the rat. J Nutr 35:425–438, 1948.
125. Rechcigl M Jr, Berger S, Loosli JK, Williams HH. Dietary protein and utilization of vitamin A. J Nutr 76:435–440, 1962.
126. Corey JE, Hayes KC. Cerebrospinal fluid pressure, growth and hematology in relation to retinol status of the rat in acute vitamin A deficiency. J Nutr 102:1585–1594, 1972.

127. Evans HM, Bishop KS. On an invariable and characteristic disturbance of reproductive function in animals reared on a diet poor in fat soluble vitamin A. Anat Rec 23:17, 1922.
128. Appling DR, Chytil F. Evidence of a role for retinoic acid (vitamin A-acid) in the maintenance of testosterone production in male rats. Endocrinology 108:2120–2123, 1981.
129. Thompson JN, Howell JMcC, Pitt GAJ, McLaughlin EI. The biological activity of retinoic acid in the domestic fowl and the effects of vitamin A deficiency on the chick embryo. Brit J Nutr 23:471–490, 1969.
130. Fidge NH, Shiratori T, Ganguly J, Goodman, DS. Pathways of absorption of retinal and retinoic acid in the rat. J Lipid Res 9:103–109, 1968.
131. Das RC, Sarada K, Murthy SK, Ganguly J. Isolation of a receptor for retinol from the oviduct magnum of laying hens. Indian J Biochem Biophys 15:251–254, 1978.
132. Mehta G, Moon RC. Hormonal regulation of retinoic acid binding proteins in the mammary gland. Biochem J 200:591–595, 1981.
133. Lucy JA. Some possible roles for vitamin A in membranes: Micelle formation and electron transfer. Amer J Clin Nutr 22:1033–1044, 1969.
134. Fell HB, Dingle JT. Studies on the mode of action of excess vitamin A. 6. Lysosomal protease and the degradation of cartilage matrix. Biochem J 87:403, 1967.
135. Pitt GAJ. Vitamin A. In: Isler O, ed. Carotenoids. Basel and Stuttgart, Birkhauser Verlag, pp718–741, 1971.
136. Goodall AH, Fisher D, Lucy JA. Cell fusion, haemolysis and mitochondrial swelling induced by retinol and derivatives. Biochim Biophys Acta 595:9–14, 1980.
137. Dingle JT, Fell HB, Goodman DS. The effect of retinol and of retinol binding protein on embryonic skeletal tissue in organ culture. J Cell Sci 11:393, 1972.
138. Roels OA, Anderson OR, Lui NST, Shah DO, Trout ME. Vitamin A and membranes. Amer J Clin Nutr 22:1020–1032, 1969.
139. Adhikari HR, Mullick RS, Vakil UK, Screenivasan A. Alterations in structure and function relationship in cellular and subcellular membranes in vitamin A-deficient rat. World Rev Nutr Diet 31:107–113, 1978.
140. Elias PM, Friend DS. Vitamin A-induced mucous metaplasia. An in vitro system for modulating tight and gap junction differentiation. J Cell Biol 68:173–188, 1976.
141. Dion LD, Blalock JE, Gifford GE. Retinoic acid and the restoration of anchorage dependent growth to transformed mammalian cells. Exp Cell Res 17:15–22, 1978.
142. Adamo S, De Luca LM, Akalovsky I, Bhat PV. Retinoid-induced adhesion in cultured transformed mouse fibroblasts. J Nat Cancer Inst 62:1473–1478, 1979.
143. Yaar M, Stanley JR, Katz SI. Retinoic acid delays the terminal differentiation of keratinocytes in suspension culture. J Invest Dermatol 76:363–366, 1981.
144. Meeks RG, Zaharevitz D, Chen RF. Membrane effects of retinoids: Possible correlation with toxicity. Arch Biochem Biophys 207:141–147, 1981.
145. De Luca LM, Bhat PV, Sasak W, Adamo S. Biosynthesis of phosphoryl and glycosyl phosphoryl derivatives of vitamin A in biological membranes. Fed Proc 38:2535–2539, 1979.
146. Jones G. The Intracellular Distribution of Vitamin A in Rat Liver. Ph.D. thesis. Univ of Liverpool, 1971.
147. Berman ER, Segal N, Feeney L. Subcellular distribution of free and esterified forms of vitamin A in the pigment epithelium of the retina and in liver. Biochim Biophys Acta 572:167–177, 1979.
148. Roseman S. The synthesis of complex carbohydrates by multiglycosyl transferase systems and their potential function in intracellular adhesion. Chem Phys Lipids 5:270–297, 1970.
149. Roth S. A molecular model for cell interactions. Annu Rev Biol 48:541, 1973.
150. Ashwell G, Morell AG. Membrane glycoproteins and recognition phenomena. Trends Biochem Sci 2:76–78, 1977.
151. Weiser MM. Intestinal epithelial cell surface membrane glycoprotein synthesis. II. Glycosyltransferases and endogenous acceptors of the undifferentiated cell surface membrane. J Biol Chem 218:2542–2548, 1973.
152. Hassell JR, Pennypecker JP, Yamada KM, Pratt RM. Changes in cell surface proteins during normal and vitamin A-inhibited chondrogenesis *in vitro*. Ann NY Acad Sci 312:406–409, 1978.
153. Pennypacker JP, Lewis CA, Hassell JR. Altered proteoglycan metabolism in mouse limb mesenchyme cell cultures treated with vitamin A. Arch Biochem Biophys 186:351–358, 1978.
154. Gallandre F, Kistler A. Inhibition and reversion of chondrogenesis by retinoic acid in rat limb bud cell cultures. Wilhelm Roux's Arch fur Entwicklungsmech Org 189:25–33, 1980.
155. Kwasigroch TE, Kochhar DM. Vitamin A alters the internal viscosity of fragments of limb-bud mesenchyme. J Embryol Exp Morphol 59:325–339, 1980.
156. Futterman S, Rollins M. The catalytic isomerization of all-*trans*-retinal to 9-*cis*-retinal and 13-*cis*-retinal. J Biol Chem 248:7773–7779, 1973.
157. Sommer A. Nutritional blindness: Xerophthalmia and keratomalacia. Oxford, Oxford Univ Press, 1982.
158. Chader GJ. Retinoids in ocular tissues: Binding proteins, transport, and mechanism of action. In: McDivitt D, ed. Cell Biology of the Eye. New York, Academic Press, pp377–433, 1982.

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Chronic Effect of TSH on Human Thyroid Tissue in Organ Culture (41538)

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Abstract. The chronic effect of TSH on thyroidal cAMP concentrations and release of thyroid hormones was investigated using human thyroid tissue in organ culture. Normal human thyroid slices were placed in HAM's F-10 synthetic culture medium in Falcon organ tissue culture dishes, and incubated at 37° in a humidified atmosphere of 5% CO_2 in air. Medium was changed everyday and daily T_3 or T_4 release was determined using concentration of T_3 or T_4 in the medium. After incubation, slices were transferred to the medium containing 10 m*M* theophylline and incubated without TSH for an additional 30 min to determine thyroidal cAMP concentrations. Thyroidal cAMP concentrations in slices incubated with 10 mU/ml of TSH increased significantly at 2, 6, and 24 hr and even on the 6th day of incubation. Daily T_3 release was significantly increased above control from the 3rd day and daily T_4 release from the 4th day to the 11th day of incubation with 10 mU/ml of TSH. Histologically, almost all follicles were structurally maintained even on the 11th day of incubation. These results suggest that both thyroidal cAMP concentrations and release of thyroid hormones are stimulated chronically by TSH. This organ culture system is useful for investigating chronic effects of various materials on human thyroid tissue.

Organ culture is an ideal method to observe chronic effects of various materials on human thyroids *in vitro* since the structure of the follicles is essential for thyroid glandular function. However, only a few studies have been reported on the function of human thyroid tissue in organ culture (1–5) and none of them studied the adenylate cyclase–cyclic AMP (AC–cAMP) system in tissues and release of hormones from tissues.

The present experiments were undertaken, therefore, to observe chronic effects of TSH on thyroidal cAMP concentrations and release of hormones using human thyroid tissue in organ culture.

Materials and Methods. *Organ culture.* Normal human thyroid tissue was obtained from tissues surrounding solitary nonfunctioning adenomas in untreated euthyroid patients. Immediately after thyroid tissues were excised, they were transported to the laboratory in HAM's F-10 synthetic culture medium (Flow Laboratories, Mclean, Va.) containing 10% fetal bovine serum (Grand Island Biological Co., Grand Island, N.Y.), penicillin (100 units/ml), and streptomycin (100 μg/ml) at 4°. Slices (1.0–1.5 mm thick) were made with a Stadie–Riggs microtome. These slices (15–20 mg) were rinsed with F-10 medium and transferred on the surface of Millipore filters in 750 μl of F-10 medium with various concentrations of bovine TSH (thytropar, Armour Pharmaceutical Co., Phoenix, Ariz.) in Falcon organ tissue culture 3037 dishes and incubated at 37° in a humidified atmosphere of 5% CO_2 in air (Fig. 1).

Medium was changed daily since the amount of medium was insufficient for the size of slices to obtain good culture conditions. The change of medium was performed by transferring each slice together with the Millipore filter to another organ culture dish containing fresh culture medium.

All of the above procedures were performed under sterile conditions.

Release of T_3 and T_4 from thyroid slices. T_3 and T_4 concentrations in medium were measured by radioimmunoassay (RIA) using

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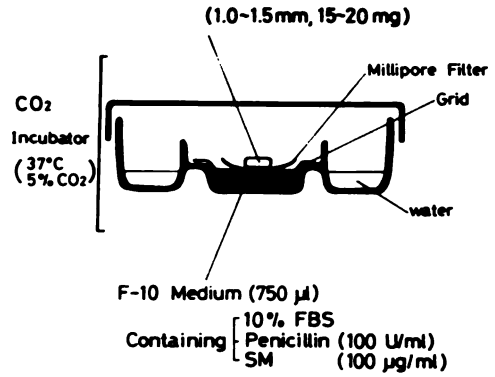

FIG. 1. Method for organ culture of normal human thyroid tissue.

commercial kits (T_3 RIA Kit and T_4 RIA Kit, Dainabot RI Laboratory, Chiba-Pref., Japan). Release of T_3 or T_4 from thyroid slices were calculated from concentrations of T_3 or T_4 in the medium and tissue weight, and expressed as picograms or nanograms per milligram weight per unit of time, respectively.

Measurement of dialyzable T_3 and T_4 concentrations. Dialysis of incubation medium was carried out as previously described (6). Six-tenths milliliter of medium was diluted with 0.6 ml of phosphate buffer containing 100 units/ml of penicillin and 100 μg/ml of streptomycin, pH 7.4, ionic strength 0.15. One-milliliter aliquots of diluted medium were then pipetted into dialysis sacs (Visking Nojax cellulose, 27/32) and were dialyzed against 5 ml of above-mentioned phosphate buffer, overnight at 37°. The concentrations of T_3 or T_4 in dialysates were measured by conventional RIA and expressed as nanograms per deciliter per milligram weight of tissue.

Thyroid cAMP concentrations. After incubation with or without TSH, slices were transferred to Falcon organ tissue culture dishes with 750 μl of F-10 medium containing 10 m*M* theophylline and incubated without TSH in a CO_2 incubator for an additional 30 min. Slices were then quickly frozen in dry ice–acetone. The frozen tissue was immediately weighed and homogenized in 1 ml ice-cold 50% acetic acid. The homogenates were centrifuged at 10,000*g* for 15 min at 4°, and the supernatants were evaporated at 50° and dissolved with 0.05 *M* sodium acetate buffer, pH 6.2. The cAMP concentrations in the buffer were measured by RIA using cyclic AMP KIT-125 (obtained from Mitsui Toatsu, Ltd., Tokyo, Japan) and expressed as picomoles per milligram weight of tissue.

Histological examination. In some experiments, tissues were fixed in isotonic buffered formalin (10%, v/v). Thin sections were cut, stained with hematoxylin and eosin, and examined under a light microscope.

Results. Daily T_3 and T_4 release into incubation medium with or without 10 mU/ml of TSH was measured until the 11th day of incubation (Fig. 2). Daily T_3 release increased

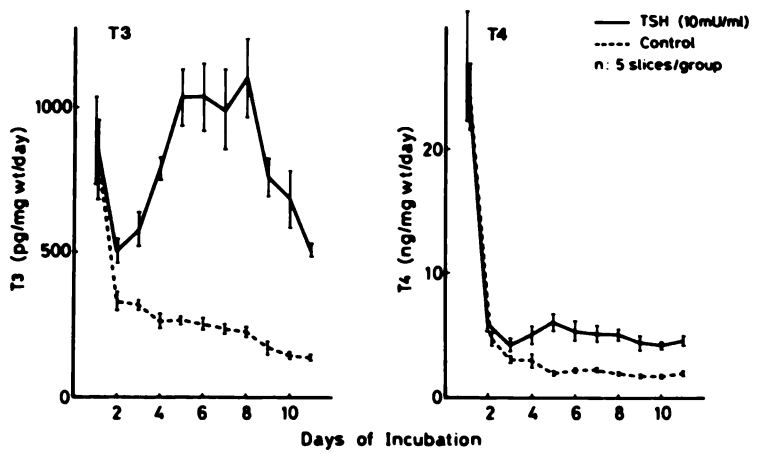

FIG. 2. Chronic effect of 10 mU/ml of TSH on T_3 and T_4 release from cultured human thyroid slices. The incubation medium was changed everyday and values represent daily T_3 and T_4 release. Bars indicate ±SE.

gradually until the fifth day of incubation with TSH, and significant differences between values in TSH-stimulated slices and control slices were observed from the third day until the 11th day of incubation. The release of T_4 paralleled that of T_3 and daily T_4 release in TSH-stimulated slices was significantly increased from the 4th day of incubation.

Cyclic AMP concentrations in slices incubated with 10 mU/ml of TSH increased significantly within 2 hr and decreased thereafter until 24 hr. However, no further significant decrease was observed after 24 hr throughout 6 days of incubation. Even after 5 days of incubation, thyroidal cAMP concentrations were significantly higher in TSH-treated slices than in controls (Fig. 3).

Dialyzable T_3 and T_4 concentrations in incubation medium were measured on the sixth day of incubation with TSH. Dialyzable T_3 and T_4 concentrations increased significantly from 1.37 ± 0.14 (control slices) to 2.81 ± 0.18 (TSH-stimulated slices) ($P < 0.005$) and from 8.5 ± 1.8 to 16.7 ± 1.5 ng/dl/mg wt ($P < 0.05$), respectively.

When thyroid slices were incubated with various doses of TSH (0.01–10.0 mU/ml), T_3 release on the fifth or sixth day of incubation increased in a dose-response manner and the increase of T_3 release was significant at TSH levels as low as 25 μU/ml (Fig. 4, Table I).

Figure 5 shows the histology of the slices after 11 days of incubation with or without 10 mU/ml of TSH. The follicular epithelium appeared healthy after 11 days of incubation with or without TSH. In slices incubated with TSH, significant reduction of colloid storage and peripheral vacuollation of colloid were observed. The height of the epithelial cells was greater in TSH-stimulated slices.

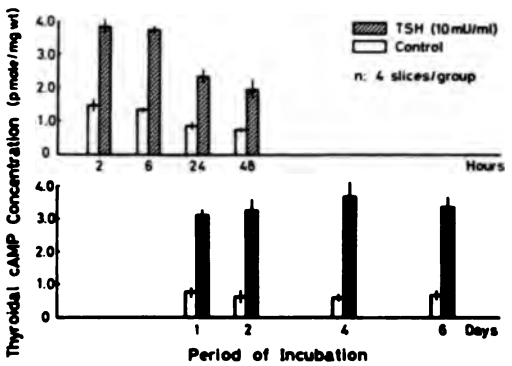

FIG. 3. Cyclic AMP concentrations in slices incubated with or without TSH for 2, 6, 24, and 48 hr (upper panel), and 1, 2, 4, and 6 days (lower panel). Thyroidal cAMP concentrations were measured after slices were transferred to Falcon organ tissue culture dishes with F-10 medium containing 10 mM theophylline and incubated without TSH in a CO_2 incubator for an additional 30 min.

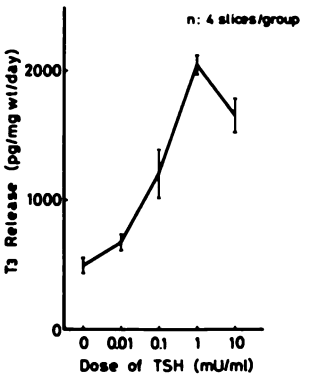

FIG. 4. Dose-response curve between doses of TSH and amounts of T_3 release on the sixth day of incubation from human thyroid slices. Bars indicate ±SE.

Discussion. In previous publications (1–5), some thyroid functions were measured in human thyroid tissue in organ culture, i.e., iodine uptake (1, 3), its distribution among thyroidal iodoaminoacid (3), and release of labeled hormone from thyroid tissue (3). Other reports describe the feasibility of applying tissue culture to postmortem tissues (2), results from organ culture of human tumors (4), and various pathological human thyroids (5). In the present experiments, release of hormones increased significantly until the 11th day of incubation with TSH. Furthermore, cyclic AMP concentrations were significantly higher in slices incubated with TSH than in controls even on the sixth day of incubation.

It has been reported that thyroid cells in tissue culture grew for similar periods of time as in the present study (7–10). In the absence of TSH, thyroid cells grew as a monolayer and have characteristics typical of epithelial cells. In the presence of TSH, thyroid cells differentiate to form two-dimensional follicles. The lumina of these follicles contain dense material reacting with thyroglobulin antisera and the cells can concentrate iodide and synthesize iodinated thyroglobulin (7, 8). Further-

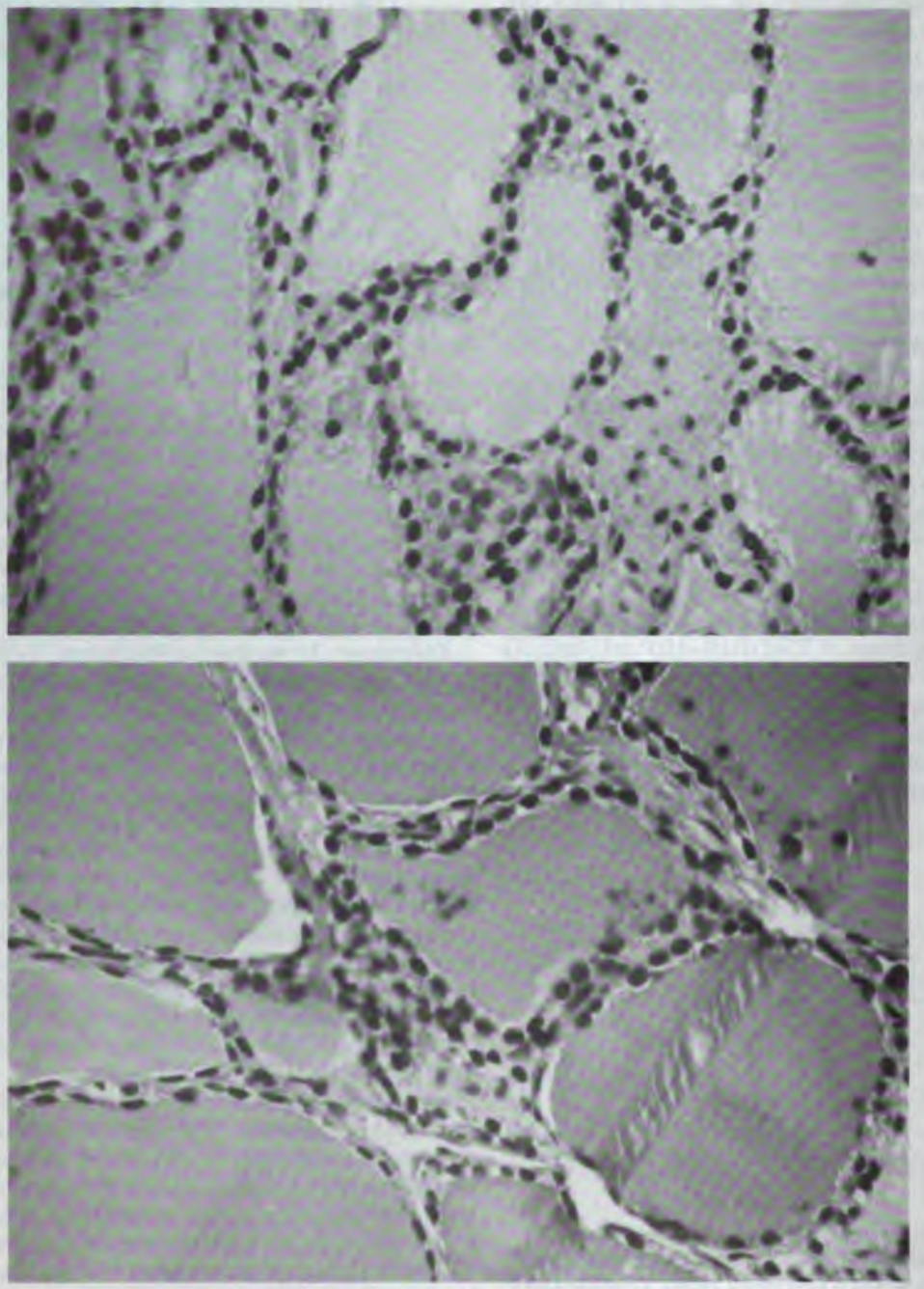

FIG. 5. Histology of the slices after 11 days of incubation with (right) or without (left) 10 mU/ml of TSH. Original magnification ×400.

TABLE I. EFFECTS OF LOW DOSES OF TSH ON T_3 RELEASE ON THE SIXTH DAY OF INCUBATION FROM HUMAN THYROID SLICES

	Dosages (μU/ml incubation medium)			
	Control	12.5	25.0	50.0
T_3 release (pg/mg wt/day)	232.13 ± 19.43[a]	300.45 ± 34.58	375.45 ± 48.90*	642.98 ± 75.56*

[a] Values shown represent the mean ± SE of the results obtained from four slices each group.
* Significantly higher than control ($p < 0.05$).

more, cAMP concentrations in cultured thyroid cells increased in response to TSH (10–12). However, a maximum increase of cAMP concentrations was observed after 10 to 30 min of stimulation (11, 12), and then cAMP concentrations declined rapidly attaining basal levels at the fourth day of culture (10). While the release of hormone from cultured thyroid cells was also increased significantly by TSH stimulation, it was maximal at 24 to 48 hr, and progressively diminished (13, 14). Thus, thyroid cells in tissue culture tend to lose their responsiveness to TSH after a period of a few days.

TSH-induced refractoriness has been reported not only in cultured thyroid cells (14), but also in studies of tissue immersed in buffer (16–18). In those reports, the effects of TSH on adenylate cyclase–cAMP system, glucose oxidation, phospholipid synthesis, organification of iodide, and colloid droplet formation were observed in thyroid slices previously incubated with TSH for several hours. On the other hand, there have been several reports of patients with TSH-producing tumors whose thyroids were continuously secreting excessive thyroid hormones indicating relative nonrefractoriness (19, 20). In the present study, using normal human thyroid slices, both thyroid cAMP concentrations and release of thyroid hormones were stimulated chronically by TSH.

The organ culture is a useful system to investigate chronic effects of various materials on human thyroid tissue under conditions independent from neural and humoral control.

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1. Hagmüller K, Leslie I. Exp Cell Res 27:396, 1962.
2. Sherwin PR, Gardner M, Richters V. Amer J Clin Pathol 43:114, 1965.
3. Hung W, Winship T, Bowen K, Houck JC. Proc Soc Exp Biol Med 122:121, 1966.
4. Roller MR, Owen SP, Heidelberger C. Cancer Res 26(Part 1), 626, 1966.
5. Nicol AG, Beck JS. Brit J Exp Pathol 49:421, 1968.
6. Uchimura J, Nagataki S, Tabuchi T, Mizuno M, Ingbar SH. J Clin Endocrinol Metab 42:561, 1976.
7. Hung W, Winship T. Proc Soc Exp Biol Med 116:887, 1964.
8. Lissitzky S, Fayet G, Giraud A, Verrier B, Torresani J. Eur J Biochem 24:88, 1971.
9. Winand RJ, Kohn LD. J Biol Chem 250:6534, 1975.
10. Verrier B, Cailla HL. Biochem Biophys Acta 428:233, 1976.
11. Kaneko Y. Horm Metab Res 8:202, 1976.
12. Rapoport B. Endocrinology 98:1189, 1976.
13. Bidey SP, Marsden P, Anderson J, Mckerron CG, Berry H. J Endocrinol 72:87, 1977.
14. Bidey SP, Marsden P, Mckerron CG, Anderson J. Biochem Biophys Res Commun 70:418, 1976.
15. Rapoport B, Adams RJ. J Biol Chem 251:6653, 1976.
16. Shuman SJ, Zor U, Chayoth R, Field JB. J Clin Invest 57:1132, 1976.
17. Field JB, Bloom G, Chou CY, Kerins ME. J Clin Invest 59:659, 1977.
18. Field JB, Dekker A, Titus G, Kerins ME, Worden W, Frumess R. J Clin Invest 64:265, 1979.
19. Kourides IA, Ridgway EC, Weintraub BD, Bigos ST, Gershengorn MC, Maloof AF. J Clin Endocrinol Metab 45:534, 1977.
20. Waldhäusl W, Bratush-Marrain P, Nowotny P, Buchler M, Forssmann WG, Lujf A, Schuster H. J Clin Endocrinol Metab 49:879, 1979.

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Calcium Regulating Hormones during the Estrous Cycle of the Rat (41539)

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Abstract. Plasma levels of calcium (Ca), phosphate (P), calcitonin (CT), parathyroid hormone (PTH), and prolactin (PRL) were measured at 8, 13, and 17 hr during the 4-day estrous cycle of the rat. Ca levels fell throughout the day during proestrus (PE) and estrus (E). In contrast Ca rose transiently during diestrus (D1, D2). P levels fluctuated inconsistently at all stages of the cycle with the exception of E where P levels were significantly higher at 13 hr. CT levels showed an increase during D1 and D2 and fell to their lowest values during E, at 13 hr. Daily fluctuations in each stage were also recorded. Variations of PTH levels during the estrous cycle were minor. PRL levels increased sharply during PE. No direct relationship between PRL secretion and CT secretion could be established. These results indicate that CT but not PTH varies specifically in relation to the estrous cycle. They suggest that there is a link between sexual hormones and CT, apparently independent of plasma calcium levels.

Among the several factors affecting calcitonin (CT) levels, sex has recently been shown to exert an important influence. For instance, Roos *et al.* (1) and Peng and Garner (2) have observed higher levels of CT in female than in male rats during aging. CT levels also vary according to the reproductive state. Circulating hormone increases during gestation (3) and lactation (4) and decreases after ovariectomy (5, 6), the lowest values are reached during estrous (7). This last finding has prompted us to investigate both calcium (Ca) and phosphate (P) levels during the estrous cycle in the rat in relation to CT, parathyroid hormone (PTH), and prolactin (PRL) fluctuations. As diurnal variations of CT levels have been reported in both male and female rats (1) blood samples were obtained at three different times in the day.

Materials and Methods. *Animals.* One hundred 3-month-old female rats (Wistar Cf) weighing 250 g were housed under controlled conditions of temperature, humidity, and light (light on 0600 to 1800 hr). Estrous cycles were monitored according to vaginal smears taken daily between 8 and 9 hr. Only animals showing three or more regular 4-day cycles were used. During the experimental period, free access to food and water was allowed. Blood samples were obtained by retroorbital sinus puncture using heparin as an anticoagulant. Plasma was immediately separated at 4° and Ca and P estimated directly. Aliquots were immediately frozen until assayed for CT, PTH, and PRL.

Calcium and phosphate estimations. Ca was estimated by atomic absorption spectrophotometry. P was measured by a colorimetric microtechnique (8).

Radioimmunoassays. CT was estimated by a sensitive assay for human CT which shows a total cross-reaction with murine CT (9, 10). In brief 50 μl of rat plasma was incubated in the presence of antiserum against human CT (HCT) at a final dilution of 1/250,000 in phosphate-buffered albumin in a total volume of 400 μl for 6 days, then 100 μl of ^{125}I-HCT (specific activity 200 μCi/μg) was added and incubation continued for another 5 days. Free and bound hormone were separated by absorption on dextran-coated charcoal. Standard curves were performed using synthetic HCT and protein effects controlled by the addition of affinity stripped plasma (50 μl) (11).

A sensitive RIA for PTH was developed using antibodies obtained in the goat (12) against 1-34 N terminal fragment of human PTH (HPTH) prepared by a solid-phase method (13) according to the sequence proposed by Niall *et al.* (14). Synthetic 1-34 HPTH was used both as standard and tracer after iodination by chloramine-T (15). Antiserum was used at a final dilution of 1/250,000 in barbital buffer, 0.05 *M*, pH 8.6, containing 17% of PTH free (affinity stripped) human

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ı. Incubation was carried out for 4 days and then tracer (specific activity 100) was added and incubation continued nore days; 100 μl of plasma sample or s quantities of standard 1-34 HPTH so- in a final volume of 500 μl were used mating unknowns or establishing stan- urves. Free and bound hormone were :ed by a modified dextran charcoal d (16). Nonspecific adsorption was lled by the incubation of each un- in the absence of antibody.

. was measured by a radioimmunoas- · rat prolactin using reagents obtained NIAMDD. Preparation of tracer (spe- tivity 150 μCi/μg) and incubation con- were carried out according to the nental protocol recommended by DD.

radioimmunoassay data were com- using a spline function (17).

istical treatment of data. Variance anal- as used for all data showing homoge- f variances (Bartlett's test) (18). In case ance heterogeneity (PRL) a *t* test was fter due reduction of the number of s of freedom (18).

ılts. *Plasma calcium levels.* Highly sig- ıt fluctuations (*F* test, $P < 0.01$) were ed during estrous cycle and during the a levels were higher at 8 hr PE as com- to 8 hr D1 and D2, and higher at 8 hr ɔmpared to 8 hr D2 ($P < 0.05$) (Table ll stages of the cycle, Ca levels declined :n morning and evening. This fall was :ant for PE between 8 and 17 hr ($P < 0.05$) and for D1 and D2 between 13 and 17 hr ($P < 0.05$) but not significant for E.

Plasma phosphate levels. P changes were similar during the day for all the stages (Table I). A significant rise was observed during E between 8 and 13 hr ($P < 0.02$).

Plasma CT levels. No differences were observed at 8 hr for all the stages (Fig. 1). High values were observed at 13 hr D1 and D2 (D1/E, $P < 0.01$; D2/E, $P < 0.02$; D1/PE, $P < 0.05$). At 17 hr D1, the increase in CT values persisted (D1/E, $P < 0.01$, D1/PE, $P < 0.02$, D1/D2, $P < 0.01$) (Fig. 1).

Plasma PTH levels. The pattern was devoid of significant fluctuations during the cycle or during the day (Fig. 1).

Plasma PRL levels. PRL levels rose during PE, with maximum values at 17 hr, 8 hr/17 hr, $P < 0.002$ (Fig. 1). No significant change was observed during the rest of the cycle; the values were very low (17 ng/ml).

Discussion. Our previous results showed that CT levels fluctuated during the estrous cycle of the rat (7). It appears from the data reported here that the fluctuations in levels of Ca are not correlated with those of CT; the physiological secretion of CT seems to be independent of variations of blood calcium levels. The data reported here also confirm our previous findings that PTH levels were not significantly modified during the cycle. Thus the restricted fluctuations in Ca levels cannot be ascribed to compensatory changes in PTH secretion. It is important to point out that we have used an N-terminal assay directed toward the biologically active part of the mol-

TABLE I. PLASMA CALCIUM AND PHOSPHATE LEVELS (mg/100 ml)

8 hours		16 hours		17 hours	
Ca	P	Ca	P	Ca	P
11.42 ± 0.12	5.18 ± 0.19	11.18 ± 0.15	5.80 ± 0.12	10.88 ± 0.13[a]	5.63 ± 0.27
11.21 ± 0.25	5.20 ± 0.16	11.02 ± 0.15	6.14 ± 0.18[d]	10.83 ± 0.17	5.48 ± 0.27
10.91 ± 0.31[a]	5.51 ± 0.26	11.19 ± 0.11	5.62 ± 0.09	10.64 ± 0.04[b]	5.45 ± 0.14
10.76 ± 0.11[a]	5.22 ± 0.30	11.21 ± 0.16	5.60 ± 0.22	10.62 ± 0.23[c]	5.61 ± 0.21

Values are the mean ± SEM for groups of five animals. PE, proestrus; E, estrus; D1, diestrus Day 1; D2, Day 2. Data were subjected to variance analysis.
ificantly different from PE, 8 hr ($P < 0.05$).
ificantly different from D1, 13 hr ($P < 0.05$).
ificantly different from D2, 13 hr ($P < 0.05$).
ificantly different from E, 8 hr ($P < 0.02$).

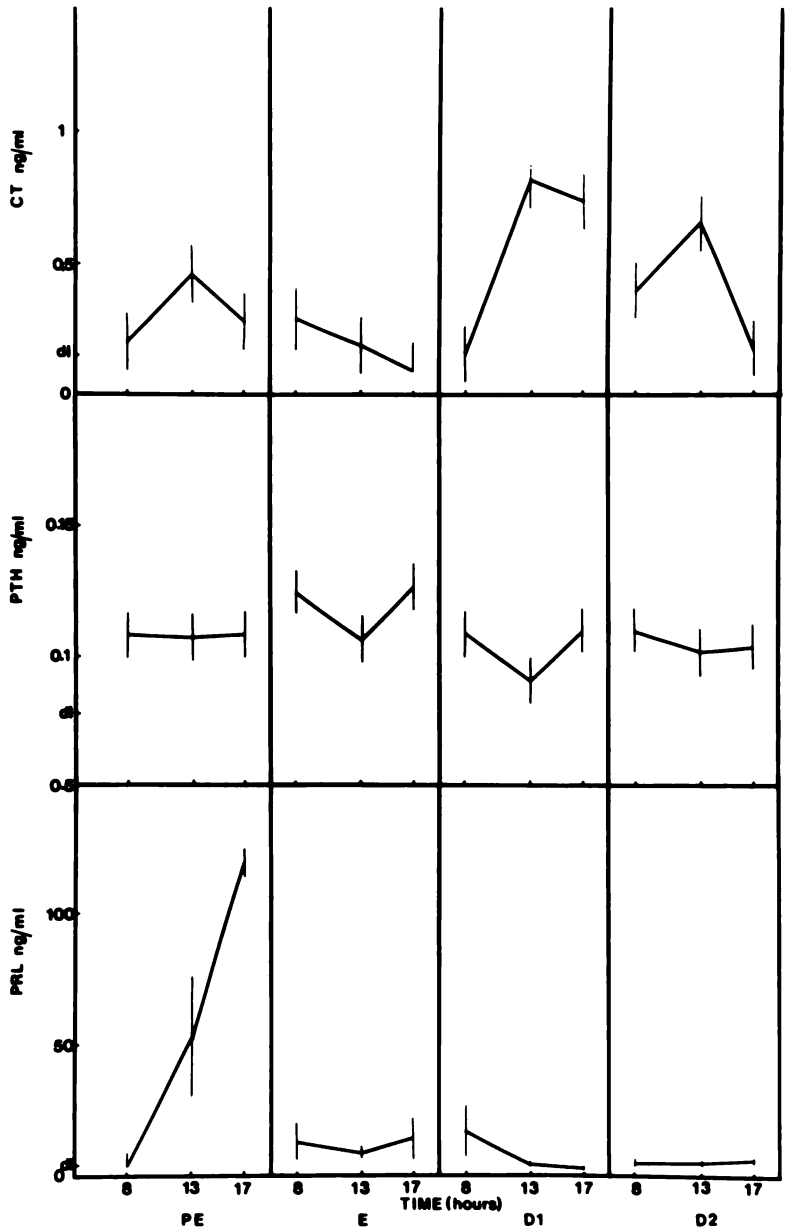

FIG. 1. Plasma PRL, CT, and PTH levels during 4-day estrous cycle. PRL levels rose during PE, reaching the highest value at 17 hr; 13 hr/17 hr, $P < 0.05$; 8 hr/17 hr, $P < 0.002$. PTH values showed no significant variations during the estrous cycle. CT values pattern differed with the hour of the day and with the day of the cycle. Higher values were observed on D1 and D2 at 13 hr: D1/E, $P < 0.001$; D2/E, $P < 0.02$; D1/PE, $P < 0.05$; and at 17 hr on D1: D1/E, $P < 0.01$; D1/PE, $P < 0.02$; D1/D2, $P < 0.01$. Values shown are the mean ± SE (vertical lines) for a group of five rats. PE, proestrus; E, estrus; D1, diestrus Day 1; D2, diestrus Day 2.

ecule. In humans, a distinct increase of PTH values has been reported during the midcycle when compared to values during the follicular and luteal phase, at which time the rise of C levels was less distinct and consistent (19 However, Baran *et al.* (20) found no change

jected to major Ca losses, the important fluctuations in CT secretion could reflect a role of the hormone during the estrous cycle.

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1. Roos BA, Cooper CW, Frelinger AL, Deftos LJ. Acute and chronic fluctuations of immunoreactive and biologically active plasma calcitonin in the rat. Endocrinology 103:2180–2186, 1978.
2. Peng TC, Garner SC. Sex difference in serum calcitonin level in rats as related to feeding, fasting and age. Endocrinology 107:289–293, 1980.
3. Garel JM, Jullienne A. Plasma calcitonin levels in pregnant and newborn rats. J Endocrinol 75:373–382, 1977.
4. Toverud SU, Cooper CW, Munson PL. Calcium metabolism during lactation: Elevated blood levels of calcitonin. Endocrinology 103:472–479, 1978.
5. Cressent M, Bouizar Z, Pidoux E, Moukhtar MS, Milhaud G. Effet de l'ovariectomie sur le taux de calcitonine plasmatique chez le rat. CR Acad Sci D 289:501–504, 1979.
6. Cressent M, Bouizar Z, Moukhtar MS, Milhaud G. Effect of ovariectomy on circulating calcitonin levels in the rat. Proc Soc Exp Biol Med 166:92–95, 1981.
7. Cressent M, Bouizar Z, Taboulet J, Moukhtar MS, Milhaud G. Calcitonin secretion during the estrous cycle of the rat. Biomedicine 33:129–130, 1980.
8. Chen PS, Toribara TY, Warner H. Microdetermination of phosphorus. Anal Chem 28:1756–1758, 1956.
9. Milhaud G, Tharaud D, Jullienne A, Moukhtar MS. Radioimmunoassay of rat calcitonin. In Taylor S, ed. Endocrinology, Proceedings, 3rd International Symposium London, 1971. London Heinemann, pp380–385, 1972.
10. Moukhtar MS, Tharaud D, Jullienne A, Raulais D, Calmettes C, Milhaud G. Immunological similarity of human and rat calcitonin confirmed by immunofluorescent methods. Experientia 29:552–555, 1973.
11. Moukhtar MS, Jullienne A, Tharaud D, Taboulet J, Milhaud G. Extraction et concentration de la calcitonine plasmatique humaine par des anticorps couplés sur support solide. CR Acad Sci D 276:3445–3448, 1973.
12. Desplan C, Jullienne A, Moukhtar MS, Raulais D, Rivaille P, Milhaud G. Isolation of circulating immunoreactive human parathyroid hormone. Comparison with a 1-34 N terminal synthetic fragment. Proc Soc Exp Biol Med 157:241–244, 1978.
13. Rivaille P, Staub JF, Jullienne A, Raulais D, Milhaud G. Strategy in the solid phase synthesis and purification of peptide hormones. Control methods applied to the active fragment of human parathyroid hormone. In Wolman Y, ed. Peptides. New York, Wiley, pp.221–225, 1975.

14. Niall HD, Saner RT, Jacobs JW, Kentmann HT, Segre GV, O'Riordan JLH, Aurbach GD, Potts JT Jr. The amino acid sequence of the amino terminal 37 residues of human parathyroid hormone. Proc Nat Acad Sci USA 71:384–388, 1974.
15. Hunter WM, Greenwood FC. Preparation of 131 I labelled human growth hormone of high specific activity. Nature (London) 194:495–496, 1962.
16. Herbert V, Lan KS, Gottlieb CW, Bleicher SJ. Coated charcoal immunoassay of insulin. J Clin Endocrinol Metab 25:1375–1384, 1965.
17. Marschner I, Erhardt F, Scriba PC. Calculation of the radioimmunoassay standard curve by spline function in RIA and related procedures in medicine. International Atomic Energy Authority, Istanbul Meeting, September 1973, Vol I:pp111–120, 1974.
18. Norman TJ, Bailey MAM. Statistical Methods in Biology. London, English Universities Press, 1959.
19. Pitkin RM, Reynolds WA, Williams GA, Hargis GK. Calcium regulating hormones during the menstrual cycle. J Clin Endocrinol Metab 47: 626–632, 1978.
20. Baran DT, Whyte MP, Haussler MR, Deftos LJ, Slatopolsky E, Avioli L. Effect of the menstrual cycle on calcium regulating hormones in the normal young woman. J Clin Endocrinol Metab 50:377–379, 1980.
21. Heath H, Sizemore GW. Plasma calcitonin in normal man. Differences between men and women. J Clin Invest 60:1135–1140, 1977.
22. Parthemore JG, Deftos LJ. Calcitonin secretion in normal human subjects. J Clin Endocrinol Metabol 47:184–188, 1978.
23. Hillyard CJ, Stevenson JC, MacIntyre I. Relative deficiency of plasma calcitonin in normal women. Lancet 1:961–962, 1978.
24. Shamonki IM, Frumar AM, Tataryn IV, Meldrum DR, Davidson BH, Parthemore JG, Judd HL, Deftos LJ. Age-related changes of calcitonin secretion in females. J Clin Endocrinol Metab 50:437–439, 1980.
25. Isaac R, Merceron RE, Caillens G, Raymond JP, Ardaillou R. Effect of parathyroid hormone on plasma prolactin in man. J Clin Endocrinol Metabol 47:18–23, 1978.
26. Isaac R, Merceron R, Caillens G, Raymond JP, Ardaillou R. Effects of calcitonin on basal and thyrotropin-releasing hormone-stimulated prolactin secretion in man. J Clin Endocrinol Metab 50:1011–1015, 1980.
27. Butcher RL, Collins WE, Fugo NW. Plasma concentration of LH, FSH, prolactin, progesterone and estradiol-17β throughout the 4-day estrous cycle of the rat. Endocrinology 94:1704–1708, 1974.
28. Tarttelin MF, Gorski RA. Variations in food and water intake in the normal and acyclic female rat. Physiol Behav 7:847–852, 1971.
29. Hirsch PF, Hagaman JR. Feeding regimen, dietary calcium and the diurnal rhythms of serum calcium and calcitonin in the rat. Endocrinology 110:961–967, 1982.
30. Rizzo AJ, Goltzman D. Calcitonin receptors in the central nervous system of the rat. Endocrinology 108:1672–1677, 1981.

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Restoration of Phosphaturia in Phosphate-Deprived Rats in the Presence of Both Parathyroid Hormone and Phosphate Infusion (41540)

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Abstract. The present study tested the hypothesis that the combination of parathyroid hormone and phosphate infusion would be phosphaturic in phosphate-deprived rats. Clearance experiments were performed in Sprague–Dawley rats fed a low-phosphate diet for 4 days. The animals were first given a phosphate infusion and then administered parathyroid hormone. The reverse experiment was also performed in a second group of rats by giving the hormone first followed by a phosphate infusion. There was no increase in phosphate excretion in response to phosphate infusion or parathyroid hormone given alone. However, the combination of parathyroid hormone and phosphate infusions, given in either order, was phosphaturic in phosphate-deprived rats.

Phosphate deprivation is characterized by avid phosphate reabsorption (1, 2) and a resistance to the phosphaturic effects of parathyroid hormone (3–5) and phosphate infusions (1, 2, 6). However, whether phosphate-deprived animals are resistant to the simultaneous infusion of phosphate and parathyroid hormone is controversial.

In a recent study, Moser *et al.* (7) demonstrated that both acute and chronic phosphate-deprived rats which were supplemented with intraperitoneal injections of phosphate to maintain serum phosphate near normal, did not develop a hypophosphaturia. These investigators attributed a critical role for serum phosphate in determining urinary phosphate excretion during phosphate deprivation. However, the administration of phosphate was also associated with a significant decrease in serum calcium levels which could have stimulated the release of parathyroid hormone, thereby producing a phosphaturia. Consequently, the authors stated that although the administration of parathyroid hormone to phosphate-deprived rats is not associated with a phosphaturia, they speculated that perhaps elevation of serum phosphate by phosphate infusion may render the renal tubule responsive to parathyroid hormone.

Previous studies in rats, however, have not supported such a notion. Bonjour *et al.* (8) observed a reduced phosphaturic response to parathyroid hormone over a wide range of plasma phosphate concentrations in conscious rats fed a low-phosphate diet. On the other hand, in a study of long-term phosphate-deprived dogs, Wong *et al.* (9) found a normal phosphaturic response with the infusion of both parathyroid hormone and phosphate.

Therefore, the present study was performed to evaluate whether parathyroid hormone is phosphaturic in phosphate-deprived rats in the presence of a phosphate infusion. We also performed the reverse experiment by giving the hormone first, followed by a phosphate infusion.

Methods. Experiments were performed on male Sprague–Dawley rats weighing 212–343 g. The animals were stabilized on a low-phosphate diet (0.07%) (ICN Pharmaceuticals, Inc., Cleveland, Ohio) for 4 days prior to the experiment. Sodium and potassium content was supplemented with NaCl and KCl as previously described (5). All animals were given food and water *ad libitum*.

On the day of the experiment, the rats were anesthetized with inactin (100 mg/kg) and prepared for clearance experiments. The animals were placed on a heated table, and body temperature was monitored with a rectal probe. A tracheotomy was performed to clear the airway and animals were allowed to breathe spontaneously. Catheters were placed in jugular veins for infusions, in the carotid

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artery for blood pressure measurement and blood sampling, and in the bladder for urine collections.

All rats underwent acute thyroparathyroidectomy (TPTX) by heat cautery followed by a 2-hr recovery period to allow for attainment of a steady state. During the second hour of recovery, the infusion of a 5% inulin solution was begun at 1.2 ml/hr for the duration of the experiment. At this point one of four protocols was performed:

Group 1: Control, P_i, P_i. The purpose of this protocol was to evaluate the effects of phosphate infusions alone within the same time frame as the group given both P_i and PTH. Following the recovery period, a 30-min clearance period was taken during which saline was also infused at 2 ml/hr. After the control clearance, the saline was subsequently replaced with a phosphate solution calculated to deliver phosphate at 2 μmole/min for the duration of the experiment. The phosphate solution was made with a 4:1 mixture of dibasic:monobasic sodium phosphate salts and adjusted to pH 7.4. Phosphate infusion proceeded for 15 min before two 30-min clearance periods were obtained. An hour was allowed to elapse followed by two additional clearance periods.

Group 2: Control, Control, PTH. The purpose of this protocol was to evaluate the effects of PTH alone within the same time frame as the group given both P_i and PTH. Following the 2-hr recovery period, a 30-min clearance period was obtained. In this group, two additional 30-min control clearance periods were taken before PTH (Synthetic, 1-34, Beckman Instruments, Palo Alto, Calif.) was administered. The hormone was given as a bolus injection (33 units/kg) followed by a maintenance infusion (60 units/kg · hr). The infusion continued for 1 hr before two 30-min clearance periods were obtained.

Group 3: Control, P_i, P_i + PTH. The purpose of this protocol was to evaluate the renal effects of parathyroid hormone given in the presence of an ongoing phosphate infusion. The protocol was similar to Group 1 with the exception that PTH was now administered during the second hour of phosphate infusion. The doses of the hormone are similar to those already described in Group 2.

Group 4: Control, PTH, PTH + P_i. The purpose of this protocol was to assess the renal effects of phosphate infusion given in the presence of parathyroid hormone. In this group the order was reversed, with parathyroid hormone being given first, followed by the infusion of phosphate.

Inulin concentrations in plasma and urine were measured by the anthrone method (10). Glomerular filtration rate (GFR) was equated with the inulin clearance. Phosphate was measured using the Chen method (11) and calcium was determined by atomic absorption spectrometry. Sodium and potassium concentrations were measured by flame photometry (Instrumentation Laboratory, Inc.).

All values are expressed as mean ± SE. Statistical comparisons were made with paired and unpaired t tests.

Results. *Control Experiments: Either P_i or PTH alone.* The effects of phosphate infusion alone in phosphate-deprived TPTX rats (Group 1) are presented in Table I. Plasma phosphate concentration increased significantly in response to the infusion of phosphate; however, no increase occurred in phosphate excretion. The high variability in the last period was due to results in one animal where phosphate excretion did increase. Plasma calcium concentration did not change

TABLE I. EFFECTS OF PHOSPHATE INFUSION ALONE IN PHOSPHATE-DEPRIVED TPTX RATS

	P_{P_i} (m*M*)	FE_{P_i} (%)	P_{Ca} (m*M*)	GFR (ml/min)	FE_{Na} (%)	BP (mm Hg)
Control	2.63 ± 0.22	0.13 ± 0.06	2.20 ± 0.29	2.1 ± 0.5	0.29 ± 0.18	129 ± 13
P_i	3.24 ± 0.30*	0.27 ± 0.19	1.98 ± 0.19	1.6 ± 0.5	0.45 ± 0.17	127 ± 14
P_i	3.81 ± 0.37*	4.13 ± 3.58	1.58 ± 0.18*	2.0 ± 0.5	1.06 ± 0.31*	111 ± 9

Note. Values are means ± SE in five animals. P_{P_i}, plasma phosphate; FE_{P_i}, fractional excretion of phosphate; P_{Ca}, plasma calcium; GFR, glomerular filtration rate; FE_{Na}, fractional excretion of sodium; BP, mean arterial blood pressure.

* Significantly different from preceding period, $p < 0.05$.

TABLE II. EFFECTS OF PARATHYROID HORMONE ALONE IN PHOSPHATE-DEPRIVED TPTX RATS

	P_{P_i} (m*M*)	FE_{P_i} (%)	P_{Ca} (m*M*)	GFR (ml/min)	FE_{Na} (%)	BP (mm Hg)
ol	2.34 ± 0.18	0.09 ± 0.01	2.45 ± 0.08	2.0 ± 0.2	0.55 ± 0.41	134 ± 3
ol	2.70 ± 0.10	0.07 ± 0.01	2.16 ± 0.04*	2.0 ± 0.2	0.99 ± 0.65	127 ± 4
	3.23 ± 0.10*	1.90 ± 0.72	2.05 ± 0.05	2.1 ± 0.2	0.53 ± 0.10	112 ± 4*

e. Values are means ± SE in six animals. PTH, parathyroid hormone. Other abbreviations as described in I.

ignificantly different from preceding period, $p < 0.05$.

ıg the first period of phosphate infusion leclined significantly with continued in- n. Glomerular filtration rate and mean ial blood pressure were not significantly ged, although blood pressure tended to luring the later stages of the experiment. ıe results of Group 2 rats which were given parathyroid hormone appear in Table II. na phosphate was significantly increased ıg infusion of the hormone, but no in- e in phosphate excretion was observed. na calcium decreased significantly in the ıd control period but upon PTH infusion ıot decline further. Glomerular filtration was not affected by parathyroid hormone. d pressure was significantly decreased, yet ined within an acceptable range (i.e.,) mm Hg).

periments in the presence of both P_i and '. In Table III, data are presented from ıp 3 wherein phosphate was infused first, wed by the additional infusion of para- ıid hormone. In response to the infusion ıosphate, there was no change in phos- e excretion. The rise in plasma phosphate ıot statistically significant. There were no ficant changes in blood pressure or glo- ılar filtration rate. Upon the addition of thyroid hormone to the infusion of phos- e there was a marked increase in frac- tional phosphate excretion from 0.09 to 22.5%. The increase in plasma phosphate was variable and did not attain statistical significance. However, plasma calcium decreased progressively. Mean arterial blood pressure was significantly lower than in the previous period, but glomerular filtration rate remained stable.

In Group 4, the reverse experiment was performed in which parathyroid hormone was administered first, followed by the combination of parathyroid hormone and phosphate infusion. The results appear in Table IV. The administration of parathyroid hormone alone did not elicit any significant changes in the parameters evaluated. Fractional phosphate excretion was not significantly increased and the value of approximately 2% was similar to that observed in the time control group (Table II). The initiation of phosphate infusion in the presence of an ongoing infusion of parathyroid hormone resulted in increases in fractional excretion of phosphate from 2.4 to 26.4%. Plasma phosphate also increased significantly; however, in this group there were no significant changes in plasma calcium. Mean arterial blood pressure was significantly lower in this period, as had been observed in the previous group. Nonetheless, glomerular filtration rate remained stable.

In these experiments, volume expansion

TABLE III. EFFECTS OF PARATHYROID HORMONE IN THE PRESENCE OF PHOSPHATE INFUSION IN PHOSPHATE-DEPRIVED TPTX RATS

	P_{P_i} (m*M*)	FE_{P_i} (%)	P_{Ca} (m*M*)	GFR (ml/min)	FE_{Na} (%)	BP (mm Hg)
ol	2.40 ± 0.13	0.09 ± 0.01	1.91 ± 0.12	2.0 ± 0.2	0.09 ± 0.03	140 ± 9
	2.65 ± 0.17	0.09 ± 0.01	1.59 ± 0.09*	2.0 ± 0.1	0.36 ± 0.09*	131 ± 9
PTH	3.01 ± 0.37	22.5 ± 6.4*	1.21 ± 0.10*	2.2 ± 0.1	0.75 ± 0.11*	110 ± 9*

e. Values are means ± SE in eight animals. Other abbreviations as described in Table I.
ignificantly different from preceding period, $p < 0.05$.

TABLE IV. EFFECTS OF PHOSPHATE INFUSION IN THE PRESENCE OF PARATHYROID HORMONE IN PHOSPHATE-DEPRIVED TPTX RATS

	P_P (mM)	FE_P (%)	P_{Ca} (mM)	GFR (ml/min)	FE_{Na} (%)	BP (mm Hg)
Control	2.80 ± 0.25	0.78 ± 0.35	1.95 ± 0.02	1.7 ± 0.1	0.47 ± 0.36	139 ± 3
PTH	2.96 ± 0.13	2.35 ± 1.79	2.00 ± 0.08	2.3 ± 0.1	0.31 ± 0.12	138 ± 5
PTH + P_i	3.72 ± 0.20*	26.4 ± 9.9*	1.87 ± 0.03	2.3 ± 0.08	0.52 ± 0.23	121 ± 6*

Note. Values are means ± SE in six animals. Other abbreviations as described in Table I.
* Significantly different from preceding period, $p < 0.05$.

was minimized, as evident from the fractional excretion of sodium which was less than 1% in all groups.

Discussion. The present study tested the hypothesis that the combination of parathyroid hormone and phosphate infusion would be phosphaturic in rats acutely phosphate deprived. There was no increase in phosphate excretion in response to phosphate infusion or parathyroid hormone given alone. However, in the presence of both factors given in either order, a marked phosphaturia was evident.

Our findings confirm the results in dogs, but differ from previous studies in rats. Wong *et al.* (9) reported, in a recent study involving long-term (30–60 day) phosphate-deprived dogs, that a normal phosphaturic response was observed with the infusion of both parathyroid hormone and phosphate. On the other hand, Bonjour *et al.* (8) observed a reduced phosphaturic response to parathyroid hormone over a wide range of plasma phosphate concentrations, induced by phosphate infusions, in rats fed a low-phosphate diet.

There are, however, several fundamental differences between the present study and the study of Bonjour *et al.* (8) in rats which should be highlighted. Although the diet utilized by Bonjour *et al.* contained more phosphate (0.2%) than the one used in the present study (0.07%), the rats were phosphate-deprived for 7 days as compared to our 4-day phosphate deprivation. Troehler *et al.* (2) have shown that the effects of phosphate-deprivation are evident within 3 days. However, it is possible that the slightly longer period of phosphate deprivation could have resulted in a more severe condition of phosphate depletion, which may explain their observation of a persistent resistance to parathyroid hormone. Second, the previous experiments were performed in conscious, chronic (48-hr) TPTX rats, whereas the present studies were in anesthetized, acute TPTX rats. Finally, different forms of parathyroid hormone were utilized in both studies. Bonjour *et al.* used a TCA extract (190–250 IU/mg from Wilson Laboratories) and infused 2.5 IU/rat · hr, whereas we infused a synthetic hormone (1-34, Beckman Instruments, 6000 IU/mg) in both a bolus (33 IU/kg) and a sustaining (60 IU/kg · hr) infusion. It is possible that the different hormone preparations and doses utilized contributed to the differences in response.

Wong *et al.* (9) suggested that the combination of phosphate and parathyroid hormone infusions restored intracellular phosphate levels and hence led to a phosphaturia. The relationship of phosphate infusion to plasma phosphate and the intracellular phosphate pools is not clear. We observed a phosphaturia in the presence of modest infusions of phosphate (2 μmole/min). Indeed, in the group given phosphate first (Group 3) a phosphaturia ensued in the absence of significant increases in plasma phosphate (Table III). Moreover, the effect was evident even when parathyroid hormone was administered first followed by phosphate (Group 4), a situation where the extent of intracellular phosphate pool repletion is not presumably as large. Clearly, the interrelationships between intracellular phosphate pools and the renal response to phosphaturic stimuli remain to be elucidated.

Phosphate infusions may have altered the pattern of segmental phosphate reabsorption along the nephron. The nephron sites of action of parathyroid hormone have been localized to the proximal convoluted and straight segments and along the accessible dis-

tal convoluted tubule (12, 13). A preliminary study (14) recently reported that phosphate uptake into brush border membrane vesicles was decreased following phosphate infusions in phosphate-deprived rats. Taken together, it is conceivable that phosphate infusions decrease phosphate reabsorption in early sites along the nephron but, in the absence of parathyroid hormone, the increased delivery of phosphate is reabsorbed in later sites, such as the pars recta or distal tubule, resulting in no increase in urinary phosphate excretion. Therefore, in the presence of parathyroid hormone, the later sites would not reabsorb the increased delivery of phosphate leading to a phosphaturia.

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1. Steele TH, DeLuca HF. Influence of dietary phosphorus on renal phosphate reabsorption in the parathyroidectomized rat. J Clin Invest 57:867–874, 1976.
2. Troehler U, Bonjour JP, Fleisch H. Inorganic phosphate homeostasis. Renal adaptation to the dietary intake in intact and thyroparathyroidectomized rats. J Clin Invest 57:264–273, 1976.
3. Harter HR, Mercado A, Rutherford WD, Rodriguez H, Slatopolsky E, Klahr S. Effects of phosphate depletion and parathyroid hormone on renal glucose reabsorption. Amer J Physiol 227:1422–1427, 1974.
4. Steele TH. Renal resistance to parathyroid hormone during phosphorus deprivation. J Clin Invest 58:1461–1464, 1976.
5. Berndt TJ, Pfeifer JD, Knox FG, Kempson SA, Dousa TP. Nicotinamide restores phosphaturic effect of PTH and calcitonin in phosphate deprivation. Amer J Physiol 242:F447–F452, 1982.
6. Haramati A, Knox FG. Relationship between plasma phosphate and renal handling of phosphate: Studies with low phosphate diet and nicotinamide. Adv Exp Med Biol 151:41–46, 1982.
7. Moser S, Brautbar N, Gura V, Kleeman CR, Massry SG. On the mechanism of hypophosphaturia during acute and chronic dietary phosphate restriction. Min Elec Metab 5:39–48, 1981.
8. Bonjour JP, Troehler U, Preston C, Fleisch H. Parathyroid hormone and renal handling of P_i: Effect of dietary Pi and diphosphonates. Amer J Physiol 234:F497–F505, 1978.
9. Wong NLM, Quamma GA, O'Callaghan TJ, Sutton RAL, Dirks JH. Renal tubular transport in phosphate depletion: A micropuncture study. Canad J Physiol Pharmacol 58:1063–1971, 1980.
10. Fuhr I, Kaczmarczyk J, Kruttegen G. Eine einefache colomtrische methods zur inulinbestimmung fur nieren clearance untersuchgen bei stoffwechsecgesunden und diabetikern. Klin Wochenschr 33:729–730, 1955.
11. Chen PS, Toribara TY, Warner H. Microdetermination of phosphorus. Anal Chem 28:1756–1758, 1976.
12. Dennis VW, Bello-Reuss E, Robinson RR. Response of phosphate transport to parathyroid hormone in segments of rabbit nephron. Amer J Physiol 233:F29–F38, 1977.
13. Pastoriza-Munoz E, Colindres RE, Lassiter WE, Lechene C. Effect of parathyroid hormone on phosphate reabsorption in rat distal convolution. Amer J Physiol 235:F321–F330, 1978.
14. Sactor B, Cheng L, Kraus E, Spector D. Rat renal phosphate reabsorption is regulated by phosphate infusion by a PTH-independent mechanism. Fed Proc 41:1597, 1982 (Abstract).

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PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 172, 168–172 (1983)

Mortality, Hemodynamics, and Aortic Properties among Male and Female Turkeys Fed β-Aminopropionitrile (41541)

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Abstract. Broad-Breasted White turkeys were randomized into four treatment groups at 4 weeks of age in each of three trials. Group 1, males, and Group 2, females, were fed an unsupplemented (control) diet from 4 to 10 weeks of age. Group 3, males, and Group 4, females, were fed the control diet supplemented with 0.07% β-aminopropionitrile (BAPN). The experiments were terminated at 10 weeks of age. There was no mortality among male and female turkeys fed the control diet or significant differences in blood pressure, heart rate, aortic tensile strength, or aortic hydroxyproline between these males and females. Sixty-five percent of the males and 21% of the females fed BAPN died of dissecting aneurysms; blood pressure, heart rate, and *dp/dt* max were not altered as a result of feeding BAPN. Aortic tensile strength was higher in control turkeys than those fed BAPN, but males fed BAPN had the lowest value. Males fed BAPN also had the lowest aortic hydroxyproline content. Ultrastructural alterations of aortic elastic and collagen fibers were more severe in males than females fed BAPN. Aortic changes did not occur in control turkeys.

Feeding of *Lathyrus odoratous* seeds (1) or β-aminopropionitrile (2–4), the toxic principle of the sweet pea plant (5), to rats leads to the development of aortic dissecting aneurysms. Likewise, feeding of β-aminopropionitrile to turkey poults also results in the formation of aneurysms (6). Mortality due to aneurysms among male, Broad-Breasted White turkeys (BBW) fed β-aminopropionitrile varies from experiment to experiment, but generally is in the range of 50% (7). Apparently, experiments have not been formulated to specifically ascertain whether male and female turkeys are equally susceptible to the angiopathy induced by the consumption of β-aminopropionitrile. Therefore, the present paper describes the results of feeding the chemical to immature male and female turkeys as pertains to mortality, hemodynamics, aortic tensile strength, aortic ultrastructure, and aortic hydroxyproline content.

Materials and Methods. Three experiments were conducted. Since the results of all three were similar, the data were combined for this presentation. Male and female BBW turkeys, purchased from a commercial hatchery, were fed an unsupplemented diet (control) until 4 weeks of age. At this time the turkeys were randomized into four treatment groups, each group consisting of 20 turkeys. Group 1 (males) and Group 2 (females) were continued on the control diet. Group 3 (males) and Group 4 (females) were fed the control diet supplemented with 0.07% β-aminopropionitrile fumarate (BAPN). Turkeys that died during the course of the experiment were necropsied. The experiment terminated at 10 weeks of age, at which time all surviving turkeys also were necropsied. Twenty-four hours prior to sacrifice, the following parameters were determined from cannulation of the carotid artery (8) following injection of the operative site with lidocaine hydrochloride: arterial blood pressure, heart rate, and *dp/dt* max (maximum rate of blood pressure rise per second). At necropsy of surviving turkeys, a 2.9-mm ring of abdominal aorta, 1 cm caphalad to the sciatic arteries, was procured for determination of aortic tensile strength (8). A small segment of aorta immediately proximal to this area was processed for electron microscopy by fixing the tissue in 3.5% buffered glutaraldehyde, postfixing in 1% OsO_4, embedding in Araldite, and examining lead citrate- and uranyl acetate-stained thin sections with an electron microscope. The abdominal aorta remaining after procurement

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served.

TABLE I. HEMODYNAMICS OF MALE AND FEMALE TURKEYS FED BAPN

ex	Blood pressure, systolic/diastolic (mm Hg)		Heart rate (beats/min)	*dp/dt* max (mm Hg/sec)	Mortality (%)
ıale	205 ± 4.9[a]	154 ± 10.1[a]	276 ± 5.0[a]	1856 ± 146[a]	0.0 ± 0.0[a]
:male	207 ± 8.7[a]	144 ± 8.0[a]	270 ± 8.2[a]	1426 ± 72[b]	0.0 ± 0.0[a]
ale	209 ± 8.2[a]	143 ± 7.9[a]	267 ± 6.0[a]	1632 ± 85[a,b]	64.0 ± 7.3[b]
nale	201 ± 9.0[a]	148 ± 11.5[a]	275 ± 3.8[a]	1586 ± 157[a,b]	21.0 ± 8.3[c]

alues with different superscripts are significantly different ($P < 0.05$) from each other.

ıns for tensile strength and electron :opy was stripped of adventitia, l to a fine consistency, dried to a con- eight, extracted with chloroform– ıl (2:1), dried under vacuum, hydro- ith 6 *N* HCl, and hydroxyproline ıed by the method of Neuman and ə).

ts. There was no mortality among ei- le or female turkeys fed the control l there was not a sex-related difference al blood pressure, heart rate, aortic strength, or aortic hydroxyproline. r, *dp/dt* max was higher in males than emales. In contrast, 64% of the males ♭ of the females fed BAPN died of ıptures. When compared to controls, lid not influence heart rate, arterial essure, or *dp/dt* max (Table I). Aortic trength was lowest in males and in- ıte in females fed BAPN; it was high- ales and females fed the control diet I). Aortic hydroxyproline was highest ıl males and females and also females N, as compared to males fed BAPN, ıg that collagen content was higher in three groups than in males fed BAPN.

II. AORTIC TENSILE STRENGTH AND AORTIC YDROXYPROLINE AMONG MALE AND FEMALE TURKEYS FED BAPN

sex	Aortic tensile strength (g/mm^2)	Aortic hydroxyproline (μg/mg dry weight)
male	129.4 ± 6.9[a]	20.1 ± 1.2[a]
female	130.2 ± 3.6[a]	18.0 ± 1.1[a]
ıale	64.6 ± 2.8[b]	16.3 ± 0.8[b]
:male	85.7 ± 5.9[c]	19.0 ± 0.7[a]

alues with different superscripts are significantly $P < 0.05$) from each other.

The ultrastructural appearance of the aortic media varied among two of the four treatment groups. The media of males and females fed the control diet was similar. In these specimens the elastic fibers between smooth muscle cells had smooth surfaces and compact bundles of collagen fibers generally were closely associated with the elastic fibers (Fig. 1). Elastic fibers between smooth muscle cells in the media of males fed BAPN were greatly swollen and severely fragmented; collagenous fibers in the vicinity were dispersed and appeared swollen (Fig. 2). In the media of females fed BAPN, elastic fibers were slightly swollen and fragmented; collagen fibers were not dispersed (Fig. 3).

Discussion. Barnett *et al.* (6) reported that feeding of BAPN to young turkeys caused mortality due to dissecting aneurysms, and also noted the gross and microscopic resemblance of this experimentally induced condition to spontaneous aneurysms seen in field flocks of turkeys. Subsequently, it was found that the first step in the crosslinking of collagen and elastin was oxidative deamination of lysine and hydroxylysine by the enzyme lysyloxidase (10). Since BAPN inhibits the activity of this enzyme, the chemical decreases the deposition of mature collagen and elastic fibers in the aorta, and, therefore, reduces aortic tensile strength.

In deoxycorticosterone acetate salt (DOCA)-induced hypertensive male rats, administration of BAPN reduced aortic lysyloxidase and the collagen content of the vessel wall (11, 12). Decreased lysyloxidase activity was noted in the skin of aneurysm-prone mottled mice (13), a condition characterized by abnormal crosslinking of collagen and elastin.

Important to the present study was the report that tom turkeys had lower aortic levels

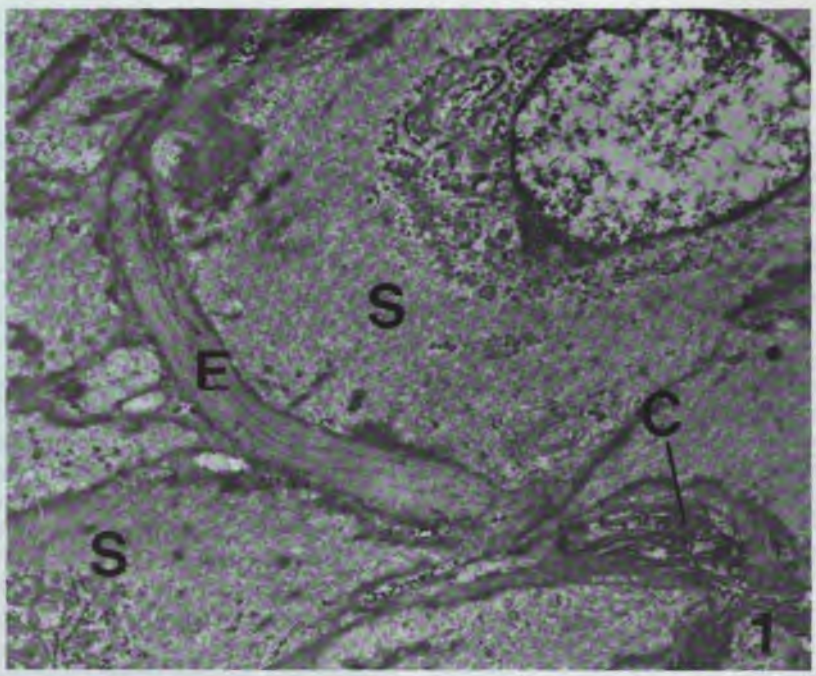

FIG. 1. The aortic media of a control male or female turkey contains elastic fibers with smooth surfaces (E) and compact bundles of collagen fibers (C) between smooth muscle cells (S). (9000×).

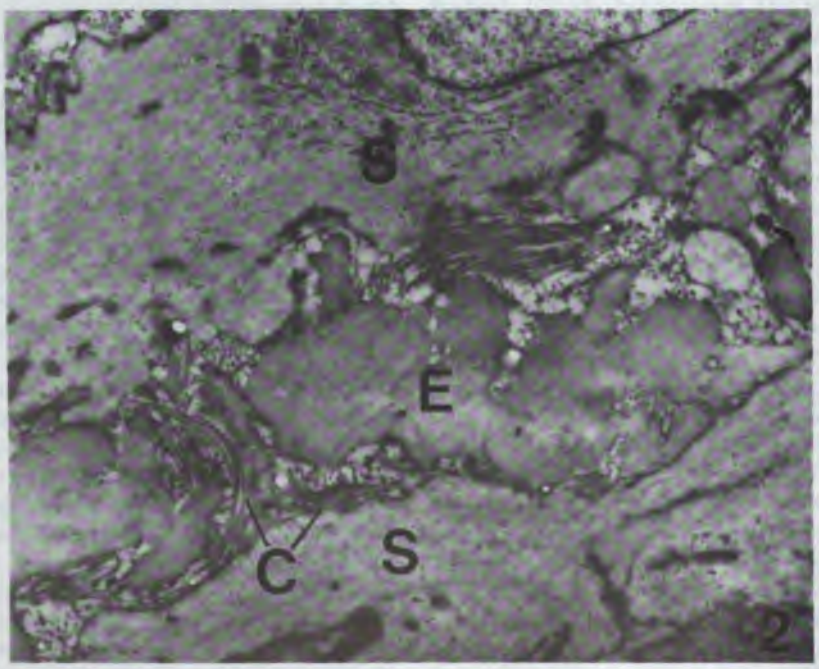

FIG. 2. In the aortic media of a male fed BAPN, an elastic fiber between smooth muscle cells (S) is greatly swollen and fragmented (E). Collagen fibers (C) are dispersed. (9000×).

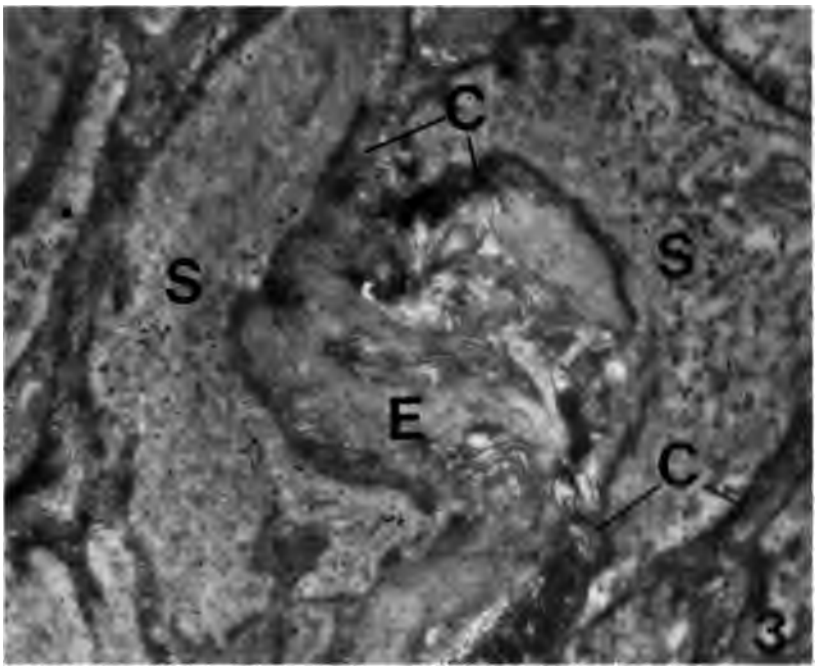

FIG. 3. An elastic fiber between smooth muscle cells (S) in the aortic media of a female fed BAPN is mildly swollen and slightly fragmented (E). Bundles of collagen fibers (C) are compact (9000×).

of lysyloxidase activity than hen turkeys (14). These results were, in the opinion of the authors, an explanation for the increased incidence of spontaneous aneurysms noted among male turkeys.

The results of the present paper indicate that the higher mortality rate from aneurysms among male turkeys fed BAPN is not associated with elevated arterial blood pressure, heart rate or *dp/dt* max since these parameters were not significantly different between males and females. Thus, the explanation for this phenomenon is probably explainable by the work of Narayanan *et al.* (14) relating to lower aortic lysyloxidase activity among male than female turkeys. This factor in addition to the lysyloxidase inhibitory activity of BAPN caused a greater accumulation of uncross-linked collagen and elastin in the male turkey aorta, and therefore, greater mortality from BAPN-induced aneurysms. This biochemical explanation is confirmed by the ultrastructural observations of the aorta reported in the present paper.

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1. Geiger BJ, Steenbock H, Parsons HT. Lathyrism in the rat. J Nutr 6:427–442, 1933.
2. Bachhuber HE, Lalich JJ, Angevine JM, Schilling EJ, Strong FM. Lathyrus factor activity of beta-aminopropionitrile and related compounds. Proc Soc Exp Biol Med 89:294–297, 1955.
3. Walker DG, Wartschatter ZJ. Histopathogenesis of aortic aneurysms in the lathyrus-fed rat. AMA Arch Pathol 61:125–135, 1956.
4. Bachhuber HE, Lalich JJ. Effect of sweet pea meal on the rat aorta. AMA Arch Pathol 59:247–255, 1955.
5. Schilling EJ, Strong FM. Isolation, structure and synthesis of a lathyrus factor from *L. odoratus*. Amer Chem Soc 77:2845–2847, 1955.
6. Barnett BJ, Bird HR, Lalich JJ, Strong FM. Toxicity of beta-aminopropionitrile for turkey poults. Proc Soc Exp Biol Med 96:[illegible]–[illegible], 1957.
7. Simpson CJ, Pritchard WR, Harms RH, Sautter JH. Skeletal and cardiovascular lesions in turkeys as

duced by feeding beta-aminopropionitrile. Exp Mol Pathol 1:305–320, 1962.

8. Simpson CF, Kling JM, Palmer RF. β-Aminopropionitrile-induced dissecting aneurysms. Treatment with propranolol. Toxicol Appl Pharmacol 16:143–153, 1970.

9. Newman RE, Logan RO. The determination of collagen and elastin in tissues. J Biol Chem 186:549–556, 1950.

10. Bornstein P, Kang A, Piez K. The nature and location of intramolecular cross-links in collagen. Proc Nat Acad Sci USA 55:417–424, 1966.

11. Sheridan PJ, Kazar LG, Benson SC. Increased lysyloxidase activity in aortas of hypertensive rats and effects of β-aminopropionitrile. Exp Mol Pathol 30:315–324, 1979.

12. Ooshima A, Midorikawa O. Increased lysyloxidase activity in blood vessels of hypertensive rats and effect of β-aminopropionitrile on arteriosclerosis. Japan Circ J 41:1337–1340, 1977.

13. Rowe DW, McGoodwin EB, Martin GR, Grahn D. Decreased lysyloxidase activity in the aneurysm-prone mottled mouse. J Biol Chem 252:939–942, 1977.

14. Narayanan AS, Sandberg LB, Jones K, Coleman SS, Bagley RA. Lysyloxidase activities of male and female turkey aortas. Exp Mol Pathol 36:107–117, 1982.

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Matrix Vesicles in Atherosclerotic Calcification[1] (41542)

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Abstract. Matrix vesicles, small extracellular membranous structures, are known to be the initial loci of calcification of cartilage, bone, and dentin. Calcification is an important complication of atherosclerosis. Using histologic, ultrastructural, and cytochemical techniques, the present study has demonstrated that matrix vesicle-like structures are involved in the calcification of atherosclerotic lesions, as well as in arterial medial calcification. In aortas from autopsied humans and from rabbits and chickens on atherogenic diets, the matrix vesicles appear to be derived from intimal and medial cellular components, mainly smooth muscle cells.

ıerosclerosis, the vascular intimal thick- which is the major cause of death in 'estern world, is commonly complicated lcification. This important complication ibutes to an increased irregularity of the ı, increased brittleness, and decreased ;ity of vessels. The degree of calcification corresponds to the severity of the ath- lerotic lesion. Morphological (1–3) and emical (4) studies characterizing crys- ɛ calcium deposition in arterial media ntima have been reported. The mecha- of calcification in atherosclerosis, how- is not well understood.

ıtrix vesicles, 100- to 700-nm extracel- nembrane-invested structures, have been n since 1967 to be the initial loci of cal- tion in skeletal tissues (5–8). More re- ', morphologic studies have suggested the vement of matrix vesicles in the calcifi- ı of aortic valves and aortic media (9– The present study, using histologic, ul- ıctural and cytochemical techniques, has nstrated that matrix vesicle-like struc- are involved in the calcification of ath- lerotic intimal lesions as well as in ar- medial calcification.

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Materials and Methods. This study utilized aortas from 30 human autopsies, 15 male New Zealand white rabbits, and 15 male white leghorn chickens. The human cases, all men over 45 years of age, included: (i) 20 cases with four portions of thoracic and abdominal aorta sampled for light microscopy, and (ii) 10 cases less than 5 hr postmortem with multiple samples of early atherosclerotic lesions taken for electron microscopy. The rabbits and chickens were each divided into the following groups: (i) three groups of three rabbits and three chickens each, fed an atherogenic diet for 1, 2, or 3 months, and (ii) three groups of two rabbits and two chickens each, fed a normal diet for 1, 2, or 3 months. The normal diet was Purina Rabbit or Chicken Chow and the atherogenic diet was composed of Purina Rabbit or Chicken Chow with 8% peanut oil and 2% cholesterol by weight mixed into the chow. Sacrifice of these animals by intravenous air injection was immediately followed by dissection and processing of their aortas.

Light microscopic study of these human and animal aortas (fixed in 3.7% neutral buffered formaldehyde solution) included the following histological stains on adjacent sections of tissue: hematoxylin and eosin; alizarin red and van Kossa for calcium and phosphate; oil red O for lipid; Verhoeff–van Gieson for elastic fibers; Gomori's trichrome for collagen and smooth muscle; Prussian blue for iron; and alcian blue–PAS for acid mucopolysaccharides. Ultrastructural study of these aortas was performed on a Phillips EM 300 electron microscope and utilized tissue which had been processed in the following ways: (i) routine—

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fixed in 4% glutaraldehyde, followed by 0.1 *M* cacodylate buffer at pH 7.4, postfixation in 1.33% symcollidine buffered osmium tetroxide (pH 7.4), dehydrated in ethanol, embedded in epoxy resin (Epon 812) (11), thin sectioned with a diamond knife on an ultramicrotome, and stained with lead citrate (12) and uranyl acetate (13); (ii) calcium localization by potassium pyroantimonate technique—fixed in potassium pyroantimonate–osmium solution, pH 7.6–7.8, for 4 hr, washed for 30 min in three changes of potassium acetate buffer, pH 7.4, routinely dehydrated and embedded, and thin sections stained only with uranyl acetate (14, 15); (iii) control for calcium localization—processed as in (ii) except that the tissue initially was incubated for 45 min in 1% osmium tetroxide containing (or, for further control, not containing) 10 m*M* ethylene glycol-bis-*N*,*N*-tetraacetic acid (EGTA), a chelating agent that removes calcium (and, to a much lesser extent magnesium, zinc, and cobalt) but not other cations, then fixed in 5% potassium pyroantimonate solution (14, 15); (iv) alkaline phosphatase cytochemical technique—using β-glycerophosphate as substrate, Tris buffer, $MgCl_2$ activator, and $Pb(NO_3)_2$ with lead phosphate as reaction product; controls included 0.2 × 0.2-mm pieces of rabbit kidney incubated and treated similarly (16); and (v) adenosine triphosphatase (ATPase) cytochemical technique—as described elsewhere, using adenosine 5-triphosphate (ATP) as substrate, Tris buffer, $MgSO_4$ activator, and $Pb(NO_3)_2$ with lead phosphate as reaction product; controls included 0.2 × 0.2-mm pieces of rabbit kidney incubated and treated similarly (16, 17).

Results and Discussion. It was found that, although there were some differences in the character of the atherosclerosis in these human, chicken, and rabbit aortas, the morphological findings associated with the presence of early calcification in the atherosclerotic lesions was similar among these three species. Details regarding the morphologic appearance of the atherosclerosis will be reported subsequently (18). Basically, the atherosclerotic lesions in the chicken aortas were more comparable to those in the human, with the initial development of an intimal fibrous plaque, composed largely of modified smooth muscle cells in an interstitium of collagen fibers, ground substance, and a few matrix vesicle-like structures. Spontaneous fibrous plaques were present in the aortas of chickens on a normal diet. Although the number of fibrous plaques did not notably increase, lipid (by light microscopy) and increasing calcium deposition (by light and electron microscopy) occurred in the plaques of all chickens fed the atherogenic diet for 1, 2, or 3 months. The rabbit aorta lesions had similarities to some small human and early chicken lesions but tended to have more the appearance of a fatty streak than a fibrous plaque containing less smooth muscle cells and more lipid-filled foam cells. Light microscopic study of calcification in these atherosclerotic lesions, using von Kossa and alizerin red methods, demonstrated focal prominent calcium deposition in all human aortic intimal lesions studied, and a lesser degree of calcification in the animal lesions. This was absent in controls but increased with increasing duration of atherogenic diet (0, 1+, 2+, and 3+ in chickens and 0, ±, 1+, and 2+ in rabbits, for control, 1, 2, and 3 months of diet, respectively, by independent estimate of two of the investigators).

Electron microscopic study of calcification in these atherosclerotic lesions demonstrated that the association of matrix vesicle-like structures with calcification was striking (Figs. 1, 2). These extracellular vesicles, in routinely processed electron microscopic sections, were generally limited by a single trilaminar membrane, varied in electron density, and occasionally contained crystalline material. They measured 100 to 700 nm in diameter. Occasional larger vesicles, distinct from the matrix vesicles and having a double-limiting membrane similar to mitochondria, were also present, however, suggesting that mitochondria may also be playing a role in the calcification process. The typical matrix vesicles were numerous (3+) in all human atherosclerotic lesions and tended to increase in number in chicken and rabbit aortas with increasing duration of atherogenic diet. Independent estimate by two of the investigators (A.T. and D.M.) indicated the number of vesicles in chickens to be 1+, 2+, 2+, and 3+ and 0, 1+, 2+, and 3+ in rabbits, for control, 1, 2, and 3 months of diet, respectively. The vesicles, particularly in human and chicken aortas, appeared to arise mainly from degen-

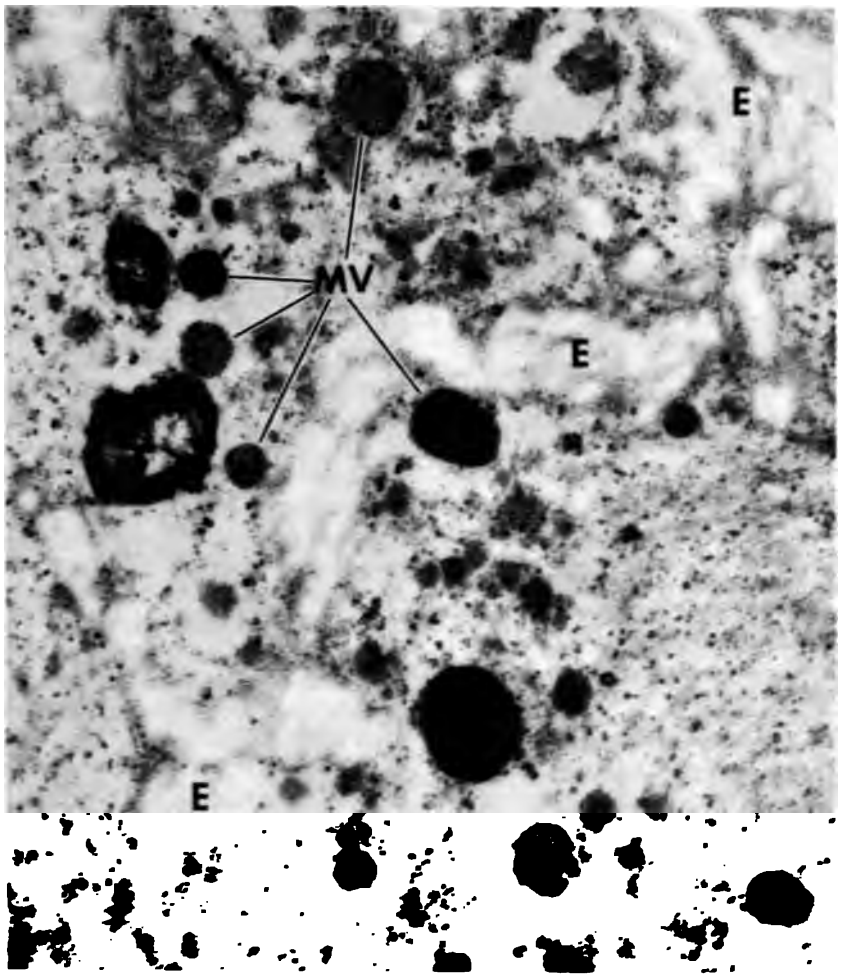

FIG. 1. Electron micrograph of atherosclerotic human aortic intima, stained by potassium pyroantimonate technique. Many membrane-limited matrix vesicles (MV) measuring 100–700 nm in diameter are located mainly in the region of elastic fibers (E). The matrix vesicles are in various stages of calcification. Calcium, indicated by the highly electron-dense pyroantimonate reaction product, is primarily deposited within the matrix vesicles but is also present in the adjacent ground substance in a much less concentrated distribution. Elastic fibers appear to be devoid of calcium deposition. ×43,000.

erated intimal and medial smooth muscle cells. Evidence for this included their location in the region of degenerated-appearing smooth muscle cells, their frequent content of fibrillary and granular material comparable to that of smooth muscle cells, their similarity to vesicles in smooth muscle cells, their occasional display of an outer component of basement membrane-like material similar to that of smooth muscle cells, and their occasionally appearing to bud from the cytoplasmic membrane or to be extruded from the cytoplasm of smooth muscle cells. Of additional interest was the frequent localization of the matrix vesicles adjacent to, or in the region of, intimal elastic fibers (Fig. 1).

Potassium pyroantimonate reaction for calcium demonstrated reaction products mainly within the matrix vesicles. (Figs. 1, 2) although some positivity was also demonstrated in other locations, such as mitochondria of some smooth muscle cells, the

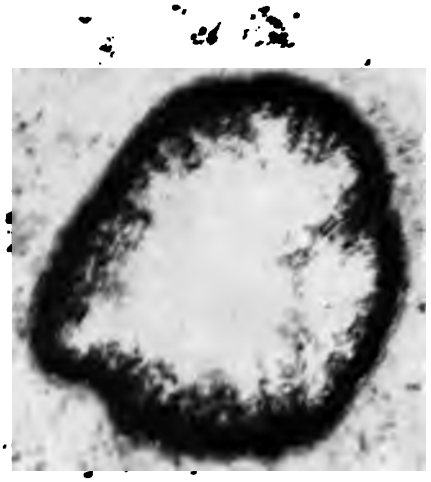

FIG. 2. Electron micrograph of atherosclerotic human aortic intima, stained by potassium pyroantimonate technique. The matrix vesicle has a single trilaminar limiting membrane and is in an intermediate stage of calcium deposition. ×79,200.

occasional extracellular mitochondria-like vesicles, interstitial foci of basement membrane-like material, and dispersed in extracellular ground substance. There was no evidence of pyroantimonate staining within elastic fibers. Although there was some variability among different lesions in the same human or animal, the overall staining reactions among the early human lesions sampled and among animals in the same experimental group were quite similar. With increasing duration of the atherogenic diet, the number of matrix vesicles not only increased but the intensity of potassium pyroantimonate reaction products also increased considerably. Independent estimate by two of the investigators (A.T. and D.M.) indicated the intensity of staining reaction in matrix vesicles to be 1+, 2+, 2+, and 3+ in chickens and ±1, 2+, 2+, and 3+ in rabbits, for control, 1, 2, and 3 months of diet, respectively. In all tissues pretreated with EGTA, essentially no potassium pyroantimonate reaction products were evident, indicating the specificity of this calcium-localization technique. Furthermore, tissues incubated without EGTA showed no decrease of potassium pyroantimonate reaction products.

Regarding the other cytochemical techniques, both ATPase and alkaline phosphatase were positive along the membranes of the matrix vesicle-like structures. In addition, smooth muscle cell mitochondria and intracytoplasmic vesicles were positive by these techniques. The positive extracytoplasmic phosphatase reactions, comparable to matrix vesicles of cartilage, supports the interpretation that the membranous structures in the atherosclerotic plaques represent matrix vesicles. Furthermore, these enzymes may play a dual role in promoting calcification: (i) by providing phosphate for nacent mineral formation, and (ii) by hydrolysis of naturally occurring inhibitors of mineral crystal formation such as pyrophosphate and ATP (19).

Calcification has long been considered an important but poorly understood complication of atherosclerosis. The significance of calcification has recently been reemphasized by reports describing the suppression of diet-induced atherosclerosis by anticalcifying agents (20). The data of this study indicate that, although other mechanisms such as mitochondrial calcification may be involved, a major mechanism in the development of atherosclerotic calcification is the deposition of calcium phosphate (mineral) within matrix vesicles apparently derived from intimal cellular components, predominantly smooth muscle cells. Comparable findings have been previously reported in the clinically less significant arterial medial calcification but to our knowledge not in the highly important process of intimal atherosclerosis. Recognition of this basic pathogenetic mechanism should open the way to several avenues (morphological, biochemical, physiological, and pharmacological) for investigating the etiology, treatment, and prevention of atherosclerotic calcification.

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1. Gardner MB, Blankenhorn DH. Aortic medial calcification. An ultrastructural study. Arch Pathol 85:397–403, 1968.
2. Yu SY. Calcification processes in atherosclerosis. Advan Exp Med Biol 43:403–425, 1974.
3. Morgan AJ. Mineralized deposits in the thoracic aorta of aged rats: Ultrastructural and electron probe x-ray microanalysis study. Exp Gerontol 15:563–573, 1980.
4. Dmitrovsky E, Boskey AL, Minick CR. Lipids associated with aortic calcification. Calcif Tissue Int 33:314, 1981.
5. Anderson HC. Electron microscopic studies of induced cartilage development and calcification. J Cell Biol 35:81–101, 1967.
6. Anderson HC. Vesicles associated with calcification in the matrix of epiphyseal cartilage. J Cell Biol 41:59–72, 1969.
7. Bonucci E. Fine structure of early cartilage calcification. J Ultrastruct Res 20:33–50, 1967.
8. Bonucci E. Fine structure and histochemistry of calcifying globulas in epiphyseal cartilage. Z Zellforsch Mikrosk Anat 103:192–217, 1970.
9. Kim KM, Valgorsky JM, Mergner WJ, Jones RT, Pendergrass RF, Trump BF. Aging changes in the human aortic valve in relation to dystrophic calcification. Human Pathol 7:47–60, 1976.
0. Kim KM. Calcification of matrix vesicles in human aortic valve and aortic media. Fed Proc 35:156–162, 1976.
11. Mollenhauer HH. Plastic embedding mixtures for use in electron microscopy. Stain Technol 39:111–114, 1964.
12. Reynolds ES. The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol 17:208–212, 1963.
13. Watson ML. Staining of tissue sections for electron microscopy with heavy metals. J Biophys Biochem Cytol 4:475–478, 1958.
14. Brighton CT, Hunt RM. Histochemical localization of calcium in growth plate mitochondria and matrix vesicles. Fed Proc 35:143–147, 1976.
15. Morris DC, Appleton J. Ultrastructural localization of calcium in the mandibular condylar growth cartilage of the rat. Calcif Tissue Int 30:27–34, 1980.
16. Matsuzawa T, Anderson HC. Phosphatases of epiphyseal cartilage studied by electron microscopic cytochemical methods. J Histochem Cytochem 19:801–808, 1971.
17. Wachstein M, Meisel E. Histochemistry of hepatic phosphatases at a physiologic pH. Amer J Clin Pathol 27:13–23, 1957.
18. Tanimura A, McGregor DH, Anderson HC. In preparation.
19. Anderson HC, Reynolds JJ. Pyrophosphate stimulation of calcium uptake into cultured embryonic bones. Fine structure of matrix vesicles and their role in calcification. Dev Biol 34:211–227, 1973.
20. Kramsch DM, Aspen AJ, Rozler LJ. Atherosclerosis: Prevention by agents not affecting abnormal levels of blood lipids. Science 213:1511–1512, 1981.

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Effect of Antibody Concentration on Opsonic Requirements for Phagocytosis *in Vitro* of *Streptococcus pneumoniae* Types 7 and 19 (41543)

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Abstract. We investigated the effect of the concentration of type-specific antibody to pneumococcal polysaccharide (PPS) on opsonic requirements for phagocytosis *in vitro* of *Streptococcus pneumonia* types 7 and 19. We measured the uptake by human neutrophils of radiolabeled *S. pneumoniae* opsonized with either complement-intact, complement-depleted (by heat inactivation), or alternative complement pathway-activated (by magnesium dichloride–ethylene glycol tetraacetic acid (MgEGTA) chelation) immune sera with varying concentrations of antibody from individuals immunized with polyvalent PPS vaccine. Increased opsonization was found with increasing concentrations of type-specific antibody in the sera. Higher concentrations of antibody were required to opsonize type 7 than type 19 bacteria, both in the presence and absence of complement activity. Type 19 bacteria were more efficiently opsonized via the alternative complement pathway than type 7. For both types, antibody and the alternative complement pathway provided most of the opsonic activity in sera with lower concentrations of type-specific antibody. At high antibody concentrations, effective opsonization occurred in heat-activated sera, indicating the requirement for complement could be overcome with sufficient amounts of antibody alone.

In spite of antimicrobial treatment, infections with *Streptococcus pneumoniae* remain a major cause of morbidity and mortality (1). Because of their polysaccharide capsules, most pneumococci are resistant to phagocytosis unless serum opsonins make them susceptible to ingestion by phagocytic cells (2). Previous studies have demonstrated that the critical opsonins for *S. pneumoniae* are type-specific antibodies to pneumococcal polysaccharide (PPS), and components of the classical and alternative complement systems (3–9). We have used a functional phagocytic assay which measures the uptake of radiolabeled *S. pneumoniae* (7, 8) to determine the effect of PPS antibody concentration on opsonization requirements for phagocytosis of *S. pneumoniae* types 7 and 19.

Materials and Methods. *Sera.* Six normal adult volunteers were immunized with 14-valent PPS vaccine (Pneumovax-MSD). Two weeks following immunization, serum was obtained and stored in aliquots at −70°. Antibody concentrations were measured by radioimmunoassay (10) (performed by Dr. G. Schiffman, Downstate Medical Center, Brooklyn, N.Y.) and by enzyme immunoassay (11) in our laboratory. Intact complement activity of the frozen aliquots was confirmed by measuring normal hemolytic complement (CH50) of ≥25 units in sera from each individual (performed by University of California, San Francisco clinical laboratories). Sera from these different individuals were utilized as either complement-intact immune serum (antibody plus classical and alternative complement pathways), serum heat-inactivated at 56° for 60 min (antibody alone), or serum chelated with a final concentration of 10 m*M* ethylene glycol tetraacetic acid with 2.5 m*M* magnesium dichloride (MgEGTA) (antibody plus alternative complement pathway) (5–9, 12). Loss of complement activity following heat inactivation was confirmed by CH50 assay. No significant differences in uptake were found between sera heat-inactivated at 56° for 30 min, 56° for 60 min, or 53° for 90 min (data not shown). Preservation of alternative complement pathway activity in MgEGTA-chelated serum was confirmed by demonstration of activated Factor B by immunoelectrophoresis following incubation of serum with opsonized zymosan (12).

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cteria. Types 7F and 19F *S. pneumoniae* :hosen for study because they are among pes which cause significant disease (13), ecause previous studies have shown they in their opsonic requirements (5, 8, 9). hilized type 7F and 19F bacteria were ıed from American Type Culture Col- n (Rockville, Md.). They were initially ı in brain heart infusion overnight at 37° umidified incubator with 5% CO_2. After / checks (plating on blood agar, and eng capsular swelling reaction with type- ic anti-PPS antiserum), the bacteria were ı in aliquots at −70° in Hanks balanced olution with 10% glycerol and 10% bo- erum albumin. The bacteria were mouse ged every 2 months to maintain viru-

radiolabel the bacteria, they were grown ıight in a synthetic medium lacking thy- ıe (14) with added tritiated methylthy- ıe (specific activity 20 Ci/mmol, New ınd Nuclear Corp., Boston, Mass.). The labeled bacteria were washed three times :alcium and magnesium-free phosphate- red saline (PBS) with centrifugation at g for 15 min, and adjusted spectropho- trically to approximately 2×10^8 bac- ml in PBS (optical density of 0.180 at ım, confirmed by microscopic counts of ɔd, diluted bacteria).

sonization. For most experiments, 100 either complement-intact or heat-inac- d immune serum, 100 μl of bacteria (ap- mately 2×10^7), and 50 μl of PBS were d to individual polypropylene vials ials, Beckman Instruments, Irvine,). For experiments with MgEGTA-che- serum, 100 μl of immune serum was ated at ambient temperature for 10 min 25 μl of 50 m*M* EGTA–12.5 m*M* $MgCl_2$ EGTA concentration 10 m*M*, $MgCl_2$:ntration 2.5 m*M*) followed by addition 0 μl of bacteria and 25 μl of PBS. The volume of the opsonic mixture was 250 ıd the percentage serum in it 40%. In experiments, the percentage serum con- ation was changed by varying the amount um added and adjusting the PBS added ɔpriately. The bacteria were opsonized in ndividual vials for 60 min on a rotator ° in an incubator with 5% CO_2. No sig- ınt differences were found in uptake of bacteria opsonized with immune sera for 15, 30, or 60 min (data not shown); 60 min was chosen to allow adequate time for alternative complement pathway activation (5). Then the bacteria were washed by adding 3 ml of PBS to each vial, and the vials centrifuged at 1600*g* for 15 min. The supernatants were discarded and the washed, opsonized bacteria resuspended in 100 μl of Hanks balanced salt solution with 0.1% gelatin (gel–HBSS).

Neutrophils. Blood from a single healthy individual was drawn into a heparinized syringe (10 units/ml), and one-third volume of 4.5% dextran in PBS added. The blood was allowed to sediment for 30 min and then the neutrophil-rich plasma was drawn off. A cell pellet was obtained by centrifuging the plasma at 120*g* for 10 min. The red cells in the pellet were lysed by adding 5 ml of 0.8% ammonium chloride for 5 min. The remaining cells, over 80% neutrophils, were washed three times with PBS with 0.2% glucose and 1 unit heparin/ml, and resuspended to a concentration of 4×10^6/ml in gel–HBSS.

Phagocytosis. Five hundred microliters of the neutrophil mixture (2×10^6 cells) was added to the washed, opsonized bacteria (2×10^7 bacteria) in each vial. The total volume of the phagocytic mixture was 600 μl, and the bacteria:neutrophil ratio was approximately 10:1. Preliminary experiments demonstrated this ratio to be optimal for demonstrating opsonic differences (data not shown). Each sample was run in quadruplicate so that leukocyte-associated and total radioactive counts could be determined in duplicate.

To allow phagocytosis, the vials were rotated at 37° for 30 min in an incubator with 5% CO_2. Experiments showed no significant further increase in uptakes at 45 or 60 min (data not shown). Phagocytosis was stopped by adding 3 ml of iced PBS. To determine leukocyte-associated counts, two of the four vials were centrifuged at 120*g* for 5 min at 4° and the supernatant discarded. The cell pellet containing neutrophils and associated bacteria was washed an additional two times with iced PBS to remove non-leukocyte-associated bacteria. Phase contrast microscopy demonstrated most of the leukocyte-associated bacteria to be ingested rather than merely adherent; this was the case when the bacteria

were opsonized with either complement-intact or heat-inactivated immune sera. To obtain total counts, the other two vials were centrifuged once at 1600*g* for 15 min which pelleted all bacteria and leukocytes.

The pellets from all vials were each resuspended in 200 μl distilled water and vortexed to lyse the cells; 3 ml scintillation fluid (Aquasol, New England Nuclear Corp., Boston, Mass.) was then added. The samples were counted with a Beckman LS 7000 scintillation counter. The bacterial uptake was calculated as

$$\% \text{ uptake} = \frac{\text{cpm in } 120 \times \text{g pellet (leukocyte-associated counts)}}{\text{cpm in } 1600 \times \text{g pellet (total counts)}} \times 100.$$

Total counts were always greater than 5000 cpm.

Data analysis. The mean counts from duplicate vials on a single day were used to calculate the bacterial uptake produced by an opsonic source (immune, heat-inactivated, or MgEGTA-chelated sera from a single individual). For most experiments, sera were assayed on at least 3 different days, and means and standard deviation determined from the uptake values for each day. The mean coefficient of variation for the assay was 22.4% between different days. An unpaired *t* test was used to calculate differences between mean uptake values.

Results. *Sera.* Antibody concentrations in postimmunization sera, as determined by radioimmunossay and enzyme immunoassay, are listed in Table I.

Effect of antibody concentration on bacterial uptake. The degree to which the concentration of type-specific PPS antibodies affects opsonization of *S. pneumoniae* types 7F and 19F in complement-intact immune serum was assessed in two ways. First, varying percentages of immune serum from a single individual were used to opsonize the bacteria. Because the opsonic mixture volume was constant, diluting the serum resulted in varying concentrations of antibody available for opsonization, although complement components were also diluted.

TABLE I. ANTIBODY CONCENTRATIONS IN POSTIMMUNIZATION SERA

Serum sample[a]	Type 7F RIA[b] (ng AbN/ml)	Type 7F EIA[c] (OD)	Type 19F RIA (ng AbN/ml)	Type 19F EIA (OD)
1	0	0	0	0
2	239	0.23	14	0.24
3	1989	0.37	139	0.53
4	3156	0.78	48	0.13
5	>5000	0.93	708	0.85
6	>5000	0.98	886	0.79
7	>5000	0.99	1036	0.87

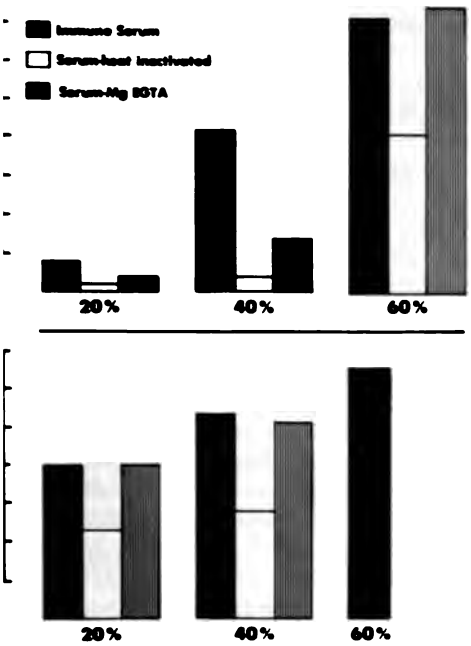
[a] Healthy adults were immunized with polyvalent pneumococcal polysaccharide vaccine, and serum samples obtained 2 weeks later. Sample 1 was from a patient with hypogammaglobulinemia.

[b] Antibodies to types 7F and 19F pneumococcal polysaccharides were measured by radioimmunoassay, and the results expressed as ng antibody nitrogen/ml.

[c] IgG antibodies to types 7F and 19F pneumococcal polysaccharides were measured by enzyme immunoassay, and the results expressed as the mean optical density from triplicate wells. The serum was diluted 1:100, and alkaline phosphatase-conjugated anti-human IgG was utilized in the assay.

In a representative experiment, type 7 bacteria were opsonized with 20, 40, and 60% immune serum containing 1989 ng antibody nitrogen (Ab N)/ml representing 5, 10, and 15×10^{-6} ng Ab N/bacterium, respectively. Increased bacterial uptake was measured with increasing antibody concentration in the opsonic mixture (solid bars, Fig. 1). A similar experiment was performed with type 19 bacteria using 20, 40, and 60% immune serum containing 47 ng Ab N/ml of type 19 PPS antibodies corresponding to 0.12, 0.24, and 0.35×10^{-6} ng Ab N/bacterium, respectively. Again, increased bacterial uptake was seen with increasing amounts of type-specific antibody (solid bars, Fig. 1). Comparing types 7 and 19, more antibody/bacterium was required for phagocytosis of type 7 bacteria than for type 19.

Dilutions of immune serum resulted in dilution of complement components as well as antibody. To study the effect of varying antibody concentrations on opsonization with constant complement concentrations, we obtained immune sera from six normal adults 2 weeks after immunization with 14-valent PPS vaccine. Because these individuals had



. 1. Effect of serum opsonizing concentration on rial uptake. Radiolabeled type 7F *S. pneumoniae* opsonized with complement-intact (solid bars), heat-vated (open bars), or MgEGTA-chelated (striped immune sera (1989 ng Ab N/ml) at concentrations , 40, and 60%, and the percentage bacterial uptake man neutrophils at 30 min measured (upper sec- Radiolabeled type 19F *S. pneumoniae* were simi- opsonized in immune serum (47 ng Ab N/ml) from er individual and the uptake measured (lower sec-

varying antibody responses to immunization, sera with different type-specific antibody amounts were available and utilized in further experiments at 40% concentration. Serum from a patient with pan-hypogammaglobulinemia (IgG = 25 mg/dl, IgM = 14 mg/dl, no type 7 or 19 antibodies by radioimmunoassay) was also studied.

For type 7, bacterial uptake was ≤10% when complement-intact immune serum containing no or minimal type-specific antibody (239 ng Ab N/ml, 1.2×10^{-6} ng Ab N/bacterium) was utilized for opsonization (solid bars, Fig. 2). Moderate uptake (40%) occurred with immune serum containing 1989 ng Ab N/ml or 10×10^{-6} ng Ab N/bacterium. The greatest uptake (65%) was measured with immune serum containing 3156 ng Ab N/ml or 15.8×10^{-6} ng Ab N/bacterium, with only minimal additional increases in uptake using immune serum with higher antibody concentrations. A relatively linear relationship between antibody concentration and uptake of opsonized type 7 bacteria was found measuring these and other serum samples (correlation coefficient = 0.91) (Fig. 3).

For type 19, bacterial uptake occurred with complement-intact immune serum containing as little as 14 ng Ab N/ml (0.07×10^{-6} ng Ab N/bacterium) of type-specific antibody (solid bars, Fig. 4). Increased uptake was noted with higher concentrations of type 19 anti-

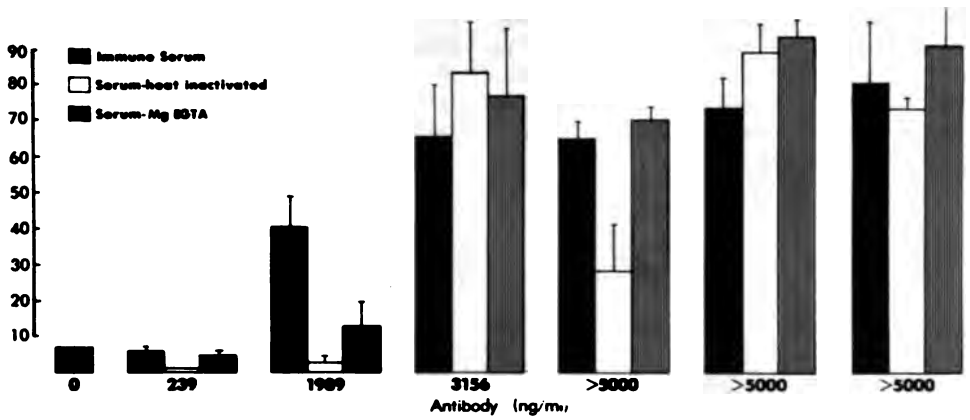


FIG. 2. Effect of antibody concentration on bacterial uptake of type 7F *S. pneumoniae*. Radiolabeled bacteria were opsonized with complement-intact (solid bars), heat-inactivated (open bars), or MgEGTA-chelated (striped bars) immune sera at 40% concentration from different individuals immunized with polyvalent pneumococcal polysaccharide vaccine, and the percentage bacterial uptake by human neutrophils measured at 30 min. Concentrations of antibody in the immune sera are given on the abscissa as ng Ab N/ml. Values for percentage bacterial uptake are expressed as mean ± 1SD for samples done in duplicate on at least 3 different days.

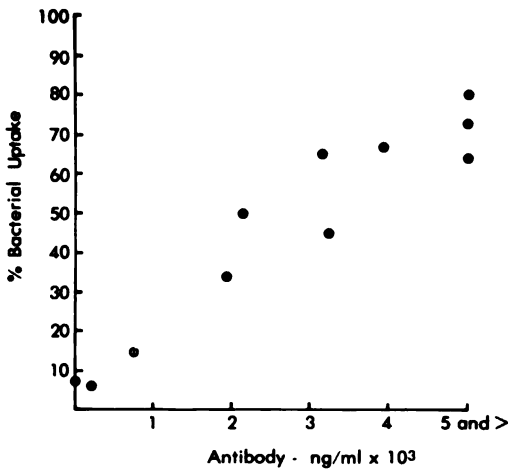


FIG. 3. Bacterial uptake correlates with concentration of type-specific antibody. Type 7F *S. pneumoniae* were opsonized with complement-intact immune sera at 40% concentration from different individuals immunized with polyvalent pneumococcal polysaccharide vaccine, and the percentage bacterial uptake by human neutrophils at 30 min measured. Bacterial uptake values are expressed as a function of ng Ab N/ml in the sera for type 7F antibodies.

body. The greatest uptake (67%) was noted at antibody concentrations of 708 ng Ab N/ml (3.5×10^{-6} ng Ab N/bacterium) or greater.

Thus, utilizing complement-intact immune sera from different individuals with varying concentrations of type-specific antibody to opsonize *S. pneumoniae* types 7 and 19, we found (1) increased opsonization, as assessed by bacterial uptake, with increasing antibody concentrations, and (2) differences in opsonization requirements for types 7 and 19, with type 7 requiring about five times more antibody than type 19 for maximal uptake.

Effect of heat inactivation on bacterial uptake. Immune sera were heat inactivated at 56° for 60 min to inactivate both the classical and alternative complement pathways. These sera were utilized to opsonize types 7 and 19 bacteria to determine the effect of antibody alone, without complement activity, on bacterial uptake. For type 7, dilutions of a single heat-inactivated immune serum containing 1989 ng Ab N/ml resulted in negligible uptake at 20 and 40% serum concentrations; however, at 60% (15×10^{-6} ng Ab N/bacterium) the uptake with heat-inactivated serum was about half that of complement-intact immune serum (open bars, Fig. 1). For type 19, there was moderate (≥20%) although reduced uptake using dilutions of heat-inactivated serum at concentrations as small as 20% (0.12×10^{-6} ng Ab N/bacterium). For both types, uptakes with complement-intact sera were higher than with heat-inactivated sera, indicating a contribution of complement to opsonic activity.

Utilizing heat-inactivated immune sera with varying antibody concentrations from different individuals we found minimal uptake (≥20%) when sera with low antibody concentrations were used to opsonize either types 7 or 19. At high antibody concentrations, however, bacterial uptake with heat-inactivated

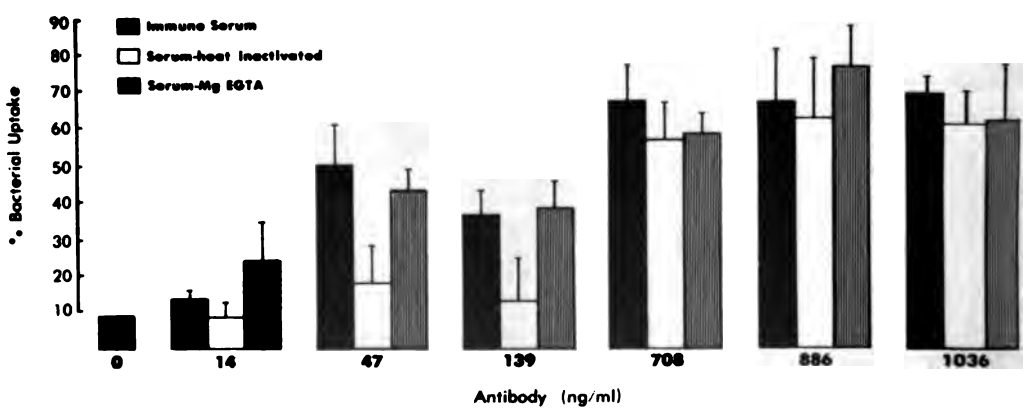


FIG. 4. Effect of antibody concentration on bacterial uptake of type 19F *S. pneumoniae*. Same as Fig. 2, except type 19F bacteria were utilized, and the amounts of antibody given on the abscissa are ng Ab N/ml for type 19F antibodies.

equaled that of complement-intact im-
e sera. For types 7, three of four heat-
ivated sera with high antibody concen-
ons (≥3156 ng Ab N/ml or 15.8×10^{-6}
.b N/bacterium) resulted in bacterial up-
equal to that of the same sera with intact
plement activity (open bars, Fig. 2). For
19, all three heat-inactivated sera with
antibody levels (≥708 ng Ab N/ml or
$< 10^{-6}$ ng Ab N/bacterium) had equal op-
c activity to that of the same comple-
t-intact sera (open bars, Fig. 4).
hus, utilizing heat-inactivated immune
we found (1) less antibody was required
psonization of type 19 than type 7; (2)
plement activity contributed to opsoni-
n of types 7 and 19, especially when lesser
entrations of type-specific antibody were
ent; and (3) opsonization equal to that
antibody and complement could be
eved with antibody alone at high concen-
ons of type-specific antibody.

ffect of MgEGTA chelation on bacterial ke. MgEGTA chelation blocks the clas-
complement pathway but allows acti-
n of the alternative pathway, permitting
surement of the opsonic capacity of the
native complement pathway in the pres-
of antibody (5–9, 12). Using dilutions of
ated immune sera to opsonize type 7 bac-
, there was minimal uptake at 20 and
serum concentrations, but uptake equal
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ie serum at 60% (striped bars, Fig. 1). At
ilutions, uptake with chelated serum was
ter than with heat-inactivated serum, in-
ting a contribution of the alternative
plement pathway to opsonic activity. For
19, uptake with chelated immune serum
equal to that with complement-intact im-
ie serum at both 20 and 40% concentra-
s, and greater than that with heat-inacti-
d serum, also suggesting alternative com-
ient pathway activity.
imilar results were obtained using Mg-
TA-chelated sera with varying concentra-
s of antibody from different individuals.
type 7 bacteria there was minimal uptake
g chelated sera containing small amounts
ntibody (1989 ng Ab N/ml (10×10^{-6} ng
N/bacterium) or less) (striped bars, Fig.
uggesting a role for antibody in alterna-
complement activation with type 7. Up-
with chelated sera was greater than that
with heat-inactivated sera, indicating an alternative complement pathway contribution to opsonic activity. At higher antibody concentrations, chelated sera provided opsonic activity equal to or greater than the same complement-intact or heat-inactivated immune sera. With type 19, bacterial uptake using chelated sera was equal to or greater than that with complement-intact immune sera regardless of antibody concentration (striped bars, Fig. 4). Uptake with three different chelated sera between 14 and 139 ng Ab N/ml was significantly ($p \leq 0.03$) greater than that with the same sera which had been heat inactivated, indicating the importance of alternative complement pathway activity for opsonization within this lower antibody range.

Therefore, measuring uptake with Mg-EGTA-chelated serum we found (1) type 19 bacteria were more efficiently opsonized via alternative complement pathway than type 7, especially at low antibody concentrations; (2) at low antibody concentrations the alternative pathway played a major role in opsonization of both types 19 and 7; and (3) the opsonic requirement for complement could be overcome by high amounts of antibody alone for both types.

Discussion. In opsonization of *S. pneumoniae*, type-specific antibodies to PPS and C3b attach to the bacteria (2–4). The presence of these molecules on the bacterial surface promotes adherence and ingestion of the organism by phagocytic cells, which have receptors for C3b and the Fc portion of certain classes of immunoglobulin. Classical complement pathway activation requires the presence of antibody, whereas the alternative pathway can be activated by the pneumococcus alone, probably via the teichoic acid constituent of its cell wall (15). The presence of antibody enhances activation of the alternative pathway, however (15, 16).

Various methods have been used to assess the opsonic requirements in man for different *S. pneumoniae* serotypes. These studies have not directly investigated the effect of antibody concentrations on opsonic requirements. Using a complement consumption assay, Fine showed that some *S. pneumoniae* types activated the alternative pathway in the absence of antibody (types 7, 12, 14, and 25), some other types could do so only if antibody was present (types 3, 4, and 8), and type 1 was

unable to do so (5). The concentration of type-specific antibody in the pooled sera studied was not quantitated.

Utilizing a chemiluminescence assay, Matthay *et al.* reported reduced opsonization of types 7, 14, and 19 with heat-inactivated immune serum, and a marked reduction with serum from which most type-specific antibody had been absorbed with the type-specific organism (9). The alternative complement pathway, assessed with MgEGTA-chelated serum, resulted in opsonic activity equal to that of immune serum for type 19, and intermediate activity for types 7 and 14. The chemiluminescence response, measured in sera of four individuals with varying type 19 antibody concentrations (0 to 765 Ab N/ml), was greater in those sera with higher antibody amounts. However, the relative effect of increasing antibody concentration on complement requirements was not studied.

Giebink and co-workers utilized pooled sera in defining opsonic requirements for several *S. pneumoniae* types with a radiolabeled bacterial uptake assay (7, 8). Their pooled sera contained 214 ng Ab N/ml of type 7, 668 Ab N/ml of type 18, 363 ng Ab N/ml of type 23, and 92 ng Ab N/ml of type 6. In dilution experiments, they found a serum concentration of 10% was adequate to result in uptakes of ≥40% for these types. With MgEGTA-chelated serum, type 18 bacteria, for which the pooled serum contained the highest antibody concentration, was opsonized at a 10% serum concentration, while types 6, 7, and 23 required 20, 40, and 60% serum, respectively, for ≥10% uptake. Markedly decreased opsonization was noted utilizing heat-inactivated serum for all except type 18, for which the serum contained the highest type-specific antibody concentration.

In the present study, we employed a modification of the method of Giebink *et al.* (7, 8) to investigate the effect of antibody amounts on opsonic requirements for types 7 and 19. Radiolabeled *S. pneumoniae* were opsonized with complement-intact, heat-inactivated, or MgEGTA-chelated sera from different individuals immunized with PPS vaccine, and the bacterial uptake by human neutrophils measured. It should be pointed out that the post-immunization sera utilized in our experiments may have contained different classes or quality of antibody than that in sera from nonimmune individuals. Furthermore, even after immunization, the quality of antibody produced by different individuals may vary. Our uptake results are not directly comparable to those of Giebink *et al.* (7, 8) because of differences in the bacteria:neutrophil ratio, duration of bacterial opsonization, and other assay conditions.

We have demonstrated increased opsonization, as assessed by bacterial uptake, with increasing concentrations of type-specific PPS antibody in the complement-intact serum used for opsonization. This is in agreement with previous studies (7–9). Minimal uptake was found using hypogammaglobulinemic serum with no PPS antibodies. The increase in uptake with antibody concentration was relatively linear for type 7 bacteria, and less so for type 19. We found greater concentrations of antibody were required for opsonization of type 7 than type 19 with complement-intact serum.

A major finding of this study is that at high concentrations of antibody the requirement for complement for opsonization of types 7 and 19 could be overcome. For type 7 this occurred at $\geq 15 \times 10^{-6}$ ng Ab N/bacterium, and for type 19 at $\geq 3.5 \times 10^{-6}$ ng Ab N/bacterium. At these high antibody levels, opsonization in heat-inactivated sera resulted in bacterial uptake equal to that of the same complement-intact immune sera in all but one sample tested. It should be noted that Giebink *et al.* found opsonic activity for only type 18 with heat-inactivated pooled sera, but it was for this type which their pooled sera contained the greatest concentration of antibody (7).

At lower antibody levels, complement activity was important for opsonization of both types 7 and 19 bacteria as demonstrated by differences found between uptakes in complement-intact versus heat-inactivated immune sera. In heat-inactivated sera, there was also increased opsonization with higher antibody concentrations, and type 7 required greater amounts of antibody for opsonization than type 19.

We have shown that type 19 bacteria were opsonized efficiently via the alternative complement pathway at all antibody concentrations tested, with uptake using MgEGTA-chelated sera equal to that with complement-intact sera. By comparing the uptake in MgEGTA-chelated sera to that in heat-inac-

tivated sera, we found the contribution of alternative complement pathway activity to opsonization was greater at lower concentrations of antibody. At high antibody concentrations, the presence or absence of alternative complement pathway activity did not appear to affect uptake.

With type 7, the presence of some antibody was required for uptake in MgEGTA-chelated serum. Although only a few samples with low antibody were measured, in all there were differences between MgEGTA-chelated and heat-inactivated serum, indicating an important role for alternative complement pathway activity. This was not the case in three out of four sera with high concentrations of antibody, where MgEGTA-chelated sera yielded uptakes equal to or greater than that in complement-intact or heat-inactivated sera. As with type 19, at high concentrations of antibody, it was antibody which provided virtually all the opsonic activity for type 7.

At least three conclusions concerning the study of host defenses against *S. pneumoniae* can be drawn from our results. First, heterogeneity exists among pneumococcal serotypes with respect to the antibody concentrations required for their effective opsonization. We found in a limited number of subjects that immunization with type 19 PPS, as part of the polyvalent vaccine, resulted in lower post-immunization antibody concentrations than type 7. Type 19 organisms, however, were more efficiently opsonized at lower antibody concentrations than type 7. Studies to determine any correlation between serotype pathogenicity, antibody response, and antibody requirements for opsonization would be desirable to determine whether this is coincidental for these two serotypes, or a more general phenomenon.

Second, complement requirements for *S. pneumoniae* opsonization may vary depending on the concentration of antibody in the sera. For example, we found opsonic activity in heat-inactivated immune sera to be equal to that in complement-intact sera when high, but not low concentrations of type-specific antibody were present. Therefore, one must take into account antibody concentration as a variable when studying complement requirements, and the results of previous studies which have not considered this variable should be reinterpreted.

Finally, although one cannot assume our *in vitro* results necessarily apply *in vivo*, our findings suggest that one might overcome opsonic defects for *S. pneumoniae* due to abnormal classical or alternative complement pathway activity by sufficiently increasing type-specific antibody concentrations. Such antibody levels were achieved for types 7 and 19 in three of six healthy individuals we immunized with polyvalent PPS vaccine. In sickle cell disease, frequent pneumococcal infections have been attributed to abnormal alternative complement pathway activity as well as to abnormal splenic function (17, 18). The efficacy of PPS vaccine immunization in sickle cell disease patients (19, 20) may result from raising type-specific antibody concentrations high enough to overcome the opsonic defect in alternative complement activity and/or splenic dysfunction (21).

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1. Klein JO. The epidemiology of pneumococcal disease in infants and children. Rev Infect Dis 3:246–253, 1981.
2. Winkelstein JA. Opsonins: Their function, identity and clinical significance. J Pediatr 82:747–753, 1973.
3. Winkelstein JA, Shin HS, Wood WB. Heat Labile opsonins to pneumococcus. III. The participation of immunoglobulins and of the alternative pathway of C3 activation. J Immunol 108:1681–1689, 1972.
4. Winkelstein JA, Smith MR, Shin HS. The role of C3 as an opsonin in the early stages of infection. Proc Soc Exp Biol Med 149:397–401, 1975.
5. Fine DP. Pneumococcal type-associated variability in alternate complement pathway activation. Infect Immunol 12:772–778, 1975.
6. Stephens CG, Williams RC, Reed WP. Classical and alternative complement pathway activation by pneumococci. Infect Immunol 17:296–302, 1977.
7. Giebink GS, Verhoef J, Peterson PK, Quie PG. Opsonic requirements for phagocytosis of *Streptococcus pneumoniae* types VI, XVIII, XXIII, and XXV. Infect Immun 18:291–297, 1977.
8. Giebink GS, Grebner JV, Kim Y, Quie PG. Serum opsonic deficiency produced by *Streptococcus pneu-*

monae and its capsular polysaccharide antigens. Yale J Biol Med 51:527–538, 1978.

9. Matthay KM, Mentzer WC, Ware DW, Preister HR, Lamers NB, Ammann AJ. Evaluation of the opsonic requirements for phagocytosis of *Streptococcus pneumoniae* serotypes VII, XIV, and XIX by chemiluminescence assay. Infect Immunol 31:228–235, 1981.

10. Schiffman G, Douglas RM, Bonner MJ, Robbins M, Austrian R. A radioimmunoassay for immunologic phenomena in pneumococcal vaccines. I. Method for the radioimmunoassay of anticapsular antibodies and comparison with other techniques. J Immunol Methods 33:133–144, 1980.

11. Gray BM. ELISA methodology for polysaccharide antigens: Protein coupling of polysaccharides for adsorption to plastic tubes. J Immunol Methods 28:187–192, 1979.

12. Des Pres RM, Bryan CS, Hawiger J, Colley DG. Function of the classical and alternate pathways of human complement in serum treated with ethylene glycol tetraacetic acid and $MgCl_2$–ethylene glycol tetraacetic acid. Infect Immunol 11:1235–1243, 1975.

13. Gray BM, Converse GM, Dillon HC. Serotypes of *Streptococcus pneumoniae* causing disease. J Infect Dis 140:950–953, 1979.

14. Sicard AM. A new synthetic medium for Diplococcus pneumoniae and its use for the study of reciprocal transformation at the am A locus. Genetics 50:31–44, 1964.

15. Winkelstein JA. The role of complement in the host's defense against *Streptococcus pneumoniae*. Rev Infect Dis 3:289–298, 1981.

16. Fearon DT, Austen KF. Current concepts in immunology. The alternative pathway of complement—A system for host resistance to microbial infection. N Engl J Med 303:259–263, 1980.

17. Johnston RB, Newman SL, Struth AG. An abnormality of the alternative complement pathway of complement activation in sickle cell disease. N Engl J Med 288:803–808, 1973.

18. Ware DW. Host defense against *Streptococcus pneumoniae*: The role of the spleen. Rev Infect Dis 3:299–309, 1981.

19. Ammann AJ, Addiego J, Ware DW, Luban BL, Smith WB, Mentzer WC. Polyvalent pneumococcal polysaccharide immunization of patients with sickle-cell anemia and patients with splenectomy. N Engl J Med 297:897–900, 1977.

20. Barrett DJ, Ammann AJ. Pneumococcal vaccine in sickle cell disease: IgG and IgM antibody response. Rev Infect Dis 3 (Suppl):S179–S182, 1981.

21. Hosea SW, Brown EJ, Hamburger MI, Frank MM. Opsonic requirements for intravascular clearance after splenectomy. N Engl J Med 304:1245–1250, 1981.

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Effects of Thyroid Hormone on the Gluconeogenic Capacity of Lipemic BHE Rats[1] (41544)

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Abstract. Gluconeogenesis and ketogenesis from a variety of substrates by isolated hepatocytes from thyroxine-treated and control 40-hr starved BHE rats were determined. Thyroid hormone treatment stimulated glucose production from lactate and inhibited glucose production from alanine, lactate plus NH_4Cl, or glutamate. β-Hydroxybutyrate production by cells incubated with glycerol was decreased in the cells from thyroxine-treated rats. Glycerol had a greater antiketogenic effect in the thyroxine-treated rats than in the control rats. Acetoacetate production from 10:1 lactate:pyruvate, lactate plus NH_4Cl, lactate plus lysine, and lactate plus ethanol was also less in the hepatocytes from the thyroxine-treated rats. Acetoacetate production from alanine was increased by thyroxine treatment. From these results it can be suggested that the metabolic abnormalities previously reported in the BHE rats can, in part, be attributable to abnormalities in endogenous thyroid hormone action and in part due to some as yet unknown regulatory process within the liver cell.

Gluconeogenesis, or the synthesis of glucose, has been shown to be controlled in part by the activity of the malate–aspartate shuttle (1–3) and in part by hormones such as glucagon (4–8), epinephrine (4–7), cortisol (9), and thyroxine (10–14). The thyroid hormone not only stimulates gluconeogenesis but also affects metabolism in other ways as well. It increases the activity of the α-glycerophosphate shuttle (15, 16), mitochondrial $Ca^{2+}Mg^{2+}$ ATPase (17), mitochondrial oxygen consumption (14, 16, 18–20), the activity of adenine nucleotide translocase (20, 21), calcium ion movement (22–24), and lowers the phosphorylation state of the hepatic cell (25). All of these actions result in an increased turnover of metabolic fuels, particularly glucose, within the hepatic cell.

In a previous paper we reported that the normally "slower" mitochondrial activity of the BHE rat[2] could be increased by thyroxine treatment (26). Both the α-glycerophosphate and the malate–aspartate shuttles were affected as was the $Ca^{2+}Mg^{2+}$ ATPase activity. We have also reported that isolated hepatocytes from BHE rats synthesized more glucose from a variety of substrates than did hepatocytes from Wistar rats (27). Since the thyroid hormones are so important in the regulation of mitochondrial activity which, in turn, is involved in the regulation of gluconeogenesis, studies of the gluconeogenic response to this hormone by the BHE rat might assist in our understanding of how the hormone functions in the regulation of this process. Secondarily, the results of these studies would expand our understanding of how the lipemic/glycemic BHE rat differs from its normal counterpart. Thus, this paper reports on the effects of thyroxine administration on the gluconeogenic capacity of hepatocytes isolated from 40-hr starved young BHE male rats.

Materials and Methods. Two groups of male 50- to 60-day-old BHE rats[2] were used. One group was injected, sc, with 10 μg thyroxine/100 g body weight while the other group was injected with solvent only and served as the control group. The dose of thyroxine was divided into two portions administered at 12-hr intervals. Thyroxine was dissolved in 5 m*M* NaOH to a concentration of 1 mg/ml. The solution was made fresh daily.

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[2] The BHE strain originated from a cross between the Osborne–Mendel and the Pennsylvania State College strains. A colony of these rats is maintained at the University of Georgia. The rats are characterized by a genetic tendency toward maturity onset diabetes and lipemia, renal disease, and cardiovascular lesions similar to a type IV lipemic. Several recent reviews have been published which describe their biochemical characteristics (52, 53).

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TABLE I. GLUCOSE PRODUCTION BY ISOLATED HEPATOCYTES OF THYROXINE-TREATED AND CONTROL 40-hr STARVED BHE RATS[a]

Incubation medium	Treatment	
	Control	10 μg thyroxine/100 g/day
No additions, 0 time	3.08 ± 0.28 (12)[b]	1.69 ± 0.12 (12)*
No additions, 60 min	8.63 ± 0.58 (14)	4.46 ± 0.17 (10)*
Lactate, 10 m*M*	41.0 ± 4.1 (12)	52.0 ± 3.7 (12)*
Pyruvate, 10 m*M*	38.8 ± 3.8 (14)+	30.8 ± 2.5 (11)+
Lactate, 10 m*M* + pyruvate, 1 m*M*	57.5 ± 4.8 (14)+	68.7 ± 3.4 (12)+
Lactate, 10 m*M* + NH_4Cl, 5 m*M*	63.5 ± 6.3 (14)+	41.7 ± 3.4 (12)*,+
Lactate, 10 m*M* + lysine, 2 m*M*	57.8 ± 5.9 (14)+	66.4 ± 2.14 (11)+
Lactate, 10 m*M* + ethanol, 10 m*M*	23.2 ± 3.3 (13)+	11.8 ± 0.28 (11)+
Alanine, 10 m*M*	24.3 ± 3.1 (10)+	9.10 ± 1.81 (10)*,+
Glutamate, 10 m*M*	19.3 ± 5.2 (14)+	8.08 ± 0.28 (12)*,+
Glycerol, 10 m*M*	28.8 ± 2.0 (14)+	48.8 ± 1.92 (11)*
Dihydroxyacetone, 10 m*M*	121.5 ± 2.1 (14)+	115.2 ± 9.85 (12)+

[a] Cells were incubated 60 min, 37° in Krebs Henseleit buffer containing 2.5% fraction V albumin with or without added substrates in a total volume of 4 ml.

[b] Results are μmole glucose/hr/g cell wet weight, mean ± SEM. Number of observations in parentheses. Significant ($P < 0.05$) hormone treatment differences are indicated by *; significant ($P < 0.05$) differences attributable to substrate addition to or substitution of lactate are indicated by +.

After 2–3 days of treatment oxygen consumption was determined using a whole body respirometer to ascertain the effectiveness of the treatment.

After 7 days of thyroxine injection, the animals were starved for 40 hr. Isolated hepatocytes were prepared according to the Krebs–Cornell modification of the Berry and Friend technique (28, 29). Viability of the cells were ascertained by trypan blue exclusion. Preparations were considered acceptable if 90–95% of the cells excluded the dye. Cells (75 mg wet weight) were suspended in Krebs–Henseleit buffer containing 2.5% fraction V bovine serum albumin. The albumin had been dialyzed with buffer to remove the excess citrate. Cells were incubated without added substrates or with the addition of 10 m*M* lactate, 10 m*M* pyruvate, 10 m*M* alanine, 10 m*M* glutamate, 10 m*M* glycerol, 10 m*M* dihydroxyacetone or 10 m*M* lactate plus 1 m*M* pyruvate, 5 m*M* NH_4Cl, 2 m*M* lysine or 10 m*M* ethanol in a total volume of 4 ml. After 60 min of incubation at 37°, the cells were killed by the addition of 0.2 ml 60% PCA. An aliquot of the cells prior to incubation was treated with PCA to provide the initial values for glucose, ketones, and ATP. The ATP content was used as another criterion of cell viability. Preparations having initial ATP values of less than 2 μmole ATP/g wet weight of cells were discarded. Ideally, isolated hepatocytes should contain between 2 and 3 μmole of ATP/g wet weight.[3] After KOH neutralization, the glucose (30), ketone (31), and ATP (32) content of the cells were determined. Glucose, β-hydroxybutyrate and acetoacetate production were calculated as micromoles product formed per hour per gram wet weight of cells. ATP content was calculated as micromoles per gram wet weight of cells. Significant differences attributable to thyroxine treatment or to the addition to or substitution for lactate were ascertained using a *t* test for groups of unequal number (33).

Results. Most investigators have used 48-hr starved rats for their studies of gluconeogenesis. However, our thyroxine-treated BHE rats could not withstand 48 hr without food and we were thus forced to use a shorter starvation period. Nonetheless, the values obtained for glucose production compare favorably with those in the literature. Glucose production from lactate or pyruvate (Table I) was slightly higher than that reported by Cornell *et al.* for female 48-hr starved Wistar rats (28) and significantly higher than those values

[3] N. Cornell, personal communication.

rted previously (27) for 40-hr starved male tar rats. The effects of lactate plus pyru-, lysine or NH_4Cl on glucose production epatocytes from control 40-hr starved rats vise are consistent with those results re-ed by Cornell *et al.* (28). The inhibition luconeogenesis by ethanol when added to ate was similar to that reported by Krebs *l.* (34) who used a perfused liver system er than isolated hepatocytes.

'reatment with thyroxine appeared to rmalize" glucose production from some not all of the substrates used. For ex-ple, glucose production was lower in the oxine-treated rats when NH_4Cl, alanine, lutamate were added to the lactate-con-ing incubation media but was stimulated n lactate alone or glycerol were used. No r thyroxine treatment effects were ob-ed. The effect of ethanol on glucose pro-tion from lactate was more pronounced epatocytes from the thyroxine-treated rats n from the nontreated rats. The values for hepatocytes from the thyroid-treated rats e very similar to those reported previously Wistar rats (27).

'onsistent with our previous reports of el-ed β-hydroxybutyrate:acetoacetate ratios rozen clamped liver (35 and elevated ke-levels in the blood of starved rats (36), ne production (Table II) by hepatocytes n both control and thyroxine-treated rats higher than that reported by others 38) and higher than that reported for ir starved Wistar rats of the same sex and (27).

-Hydroxybutyrate production (Table II) unaffected by thyroxine treatment except n glycerol was used as the substrate. With substrate, β-hydroxybutyrate production reduced. This was in contrast to the pro-tion of glucose which was increased in the atocytes from the thyroxine-treated rats npared to the control rats. β-Hydroxybu-te production was decreased when NH_4Cl added to the lactate-containing medium when alanine, glycerol, or dihydroxyac-ne were used as substrates. β-Hydroxy-yrate production was ncreased when nol was added to the lactate-containing lium. Note again the reciprocal relation-between glucose and β-hydroxybutyrate duction.

TABLE II. KETONE PRODUCTION BY ISOLATED HEPATOCYTES OF THYROXINE-TREATED AND CONTROL 40-hr STARVED BHE RATS[a]

Incubation medium	Treatment			
	β-Hydroxybutyrate		Acetoacetate	
	Control	Thyroxine	Control	Thyroxine
No addition, 0 time	6.61 ± 0.30 (14)[b]	4.92 ± 0.26 (12)[*]	13.5 ± 0.3 (14)	11.7 ± 0.74 (12)
No additions, 60 min	11.3 ± 0.6 (14)	9.27 ± 0.40 (10)	28.2 ± 0.9 (14)[+]	25.1 ± 0.4 (10)[+]
Lactate, 10 m*M*	12.7 ± 0.9 (12)	11.6 ± 1.71 (12)	12.1 ± 0.2 (12)	8.89 ± 0.44 (12)
Pyruvate, 10 m*M*	12.3 ± 0.9 (14)	14.3 ± 1.5 (11)	21.9 ± 0.6 (14)[+]	19.0 ± 0.6 (11)[+]
Lactate, 10 m*M* + pyruvate, 1 m*M*	12.3 ± 1.0 (14)	11.4 ± 1.31 (12)	12.1 ± 0.2 (14)	9.28 ± 0.42 (12)[*]
Lactate, 10 m*M* + NH_4Cl, 5 m*M*	7.32 ± 0.9 (14)[+]	9.14 ± 0.80 (12)	19.3 ± 0.8 (14)[+]	10.6 ± 1.8 (12)[*]
Lactate, 10 m*M* + lysine, 2 m*M*	14.2 ± 1.3 (14)	12.7 ± 1.8 (11)	14.0 ± 0.4 (14)	10.1 ± 0.6 (11)[*]
Lactate, 10 m*M* + ethanol, 10 m*M*	26.2 ± 0.6 (13)[+]	25.0 ± 2.1 (11)[+]	4.30 ± 0.20 (14)[+]	2.46 ± 0.10 (11)[+,*]
Alanine, 10 m*M*	6.61 ± 0.54 (10)[+]	4.89 ± 1.41 (11)[+]	16.3 ± 0.6 (12)[+]	20.2 ± 0.6 (9)[+,*]
Glutamate, 10 m*M*	11.0 ± 0.3 (14)	11.6 ± 1.1 (12)	22.6 ± 0.3 (14)[+]	21.8 ± 0.9 (12)[+]
Glycerol, 10 m*M*	5.04 ± 1.25 (14)[+]	2.21 ± 0.17 (11)[+,*]	18.7 ± 0.1 (14)[+]	16.4 ± 0.6 (11)[+]
Dihydroxyacetone, 10 m*M*	4.44 ± 0.16 (14)[+]	3.68 ± 0.66 (11)[+]	18.7 ± 0.2 (14)[+]	17.3 ± 1.0 (12)[+]

[a] Cells were incubated 60 min, 37°, in Krebs–Henseleit buffer containing 2.5% fraction V albumin with or without the indicated substrates in a total volume of 4 ml.
[b] Results are μmole ketone/hr/g cell wet weight, mean ± SEM. Number of observations in parentheses. Significant ($P < 0.05$) differences attributable to thyroxine treatment are indicated by [*]; significant ($P < 0.05$) differences attributable to substrate addition to or substitution for lactate are indicated by [+].

Acetoacetate production was lower in hepatocytes from thyroxine-treated rats than in hepatocytes from control rats when pyruvate, NH_4Cl, lysine, or ethanol were added to the lactate-containing media and higher when alanine was the substrate used. Differences in acetoacetate production with the different additions to the incubation media were observed. All but pyruvate, alanine, and glutamate lowered the acetoacetate production by the hepatocytes. The medium having lactate as a substrate resulted in a reduction (from the basal) in acetoacetate production. Glycerol or dihydroxyacetone addition did not lower acetoacetate production to the same extent as did the addition of lactate to the incubation media.

The hepatocyte content of ATP at the beginning and end of the incubation period differed little. Thyroid treatment likewise did not affect hepatocyte ATP levels. Of the various substrates used, only glycerol affected the ATP content of these cells. Cells incubated with glycerol had less ATP (0.88 ± 0.03 vs 2.10 ± 0.11), and thyroid treatment resulted in a partial restoration in the ATP content of these hepatocytes incubated with glycerol (1.65 ± 0.09 vs 0.88 ± 0.03). The difference attributable to the thyroid treatment was probably due to the effect of this hormone on the α-glycerophosphate dehydrogenase in the mitochondria and the mitochondrial α-glycerophosphate shuttle (15, 16, 26, 40–42). While thyroid treatment affected the ATP content of the cells incubated with glycerol, it also resulted in an increase in glucose production by the hepatocytes from these rats. Since glucose production from glycerol uses ATP in the initial glycerokinase step, these observations suggest that the ADP thus produced, is, under the influence of the thyroid hormone, more rapidly translocated into the mitochondria and rephosphorylated via oxidative phosphorylation. Thyroid hormone has been shown to affect adenine nucleotide translocase (21) and probably explains why the above occurs. It would also appear that under the influence of the thyroid hormone, less "futile" cycling, i.e., fructose 6-phosphate to fructose 1,6-diphosphate to fructose 6-phosphate, occurs which would reduce the amount of ATP utilized in the production of glucose such that the cellular ATP content would increase. Soling and Kleineke (43) have proposed such an explanation for the observed species differences in the energy costs and regulation of gluconeogenesis.

Discussion. The results of the present work indicate that although thyroid hormone can stimulate glucose synthesis in BHE rats, it does so selectively rather than accelerating glucose production from all substrates uniformly. Reports in the literature indicate that thyroxine induces an increase in the activity of a number of gluconeogenic enzymes (44) as well as an increase in the conversion of alanine to glucose (11), in lactate utilization for glucose formation (10), and in the conversion of pyruvate to glucose (46). Contrary to Singh and Snyder's work (11) with Sprague–Dawley rats, thyroid hormone administered to the BHE rats inhibited alanine conversion to glucose. In addition, it inhibited the stimulatory effect of NH_4Cl on lactate conversion to glucose and the utilization of glutamate for glucose production while not affecting the stimulatory effect of lysine on lactate utilization. These differences from Wistar and Sprague–Dawley rats together with our previous report (27) of increased glucose synthesis from these same substrates above that observed in Wistar rats, suggests that injections of thyroid hormone served to "normalize" (rather than induce) the gluconeogenic process in the BHE hepatocytes. It also suggests that when thyroid-treated BHE rats are starved they will be more like the Wistar rats in their choice of body tissues catabolized for fuel. That is, instead of losing more body protein and less body fat than Wistar rats (46), the reverse will be true. These differences in gluconeogenesis from nitrogen-containing substrates lend further support to the notion that the control of both urea synthesis and glucose synthesis rests with the functional capacity of the mitochondrial membrane to exchange metabolites, ions and adenine nucleotides via the various shuttle and translocase systems. While no studies have been conducted on the hepatic ureogenic capacity of BHE rats, it has been shown that ammonia levels (in relation to urea levels) in frozen clamped tissue are higher in these rats than in Wistar rats (35). While no evidence has been obtained that anion transport is rate limiting for gluconeogenesis and ureogenesis, the intracellular calcium ion movement may

rve to regulate both processes. Calcium tivates mitochondrial fatty acid oxida-7), stimulates gluconeogenesis (48–50), general acts as a second messenger for ety of hormones including the thyroid one. Thyroid hormone enhances mito-rial calcium uptake (22–24) and in so apparently increases the exchange of ne nucleotides by the adenine nucleo-ranslocase (21). The first rate-limiting f ureogenesis is the carbamyl phosphate tase step. In the mitochondria, this en-competes with the translocase for ATP the affinity of the translocase is high, mpetition will not be as successful as if finity of the translocase is low. The cal-on may well affect this affinity and hence well determine the rate of urea synthesis. the BHE rat, there was an impairment calcium ion movement either into or f the mitochondrial compartment the ination of gluconeogenesis and ureo-s, described Meijer *et al.* (51), would not efficient. If this is true, then one might t an increase in the rate of gluconeo-s accompanied by an increase in the of ammonia in the cell. Detailed studies e needed to support such an hypothesis. ough thyroid hormone decreased glu-genesis from substrates containing ni-, it did not affect gluconeogenesis from e. In fact, thyroid hormone-treated rats en greater rates of gluconeogenesis than ontrols who, in turn, synthesized nearly nore glucose from lactate than Wistar While thyroid hormone enhancement of neogenesis from lactate is consistent with erature on thyroid hormone action, the nces due to strain suggests that while f the abnormality in the BHE rat which in a maturity onset lipemia, glycemia, erative tissue changes, and a shortened in may be attributable to some abnor-in endogenous thyroid hormone ac-n these rats and correctable by exoge-thyroid hormone administration, there mains a part which has yet to be elu-d. That part seems to be intimately re-to the interplay between the regulation redox state and the phosphorylation f the cytosolic and mitochondrial com-ents. This suggestion arises from the ob-ions of raised redox and phosphoryla-tion states in frozen clamped livers (35) and from the current work which showed elevations in glucose production when the lactate:pyruvate ratio was manipulated. Future work will need to focus on this aspect of intermediary metabolism in the BHE rat compared to the Wistar rat.

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1. Williamson JR. Role of anion transport in the regulation of metabolism. In: Hanson RW, Mehlman MA, eds. Gluconeogenesis: Its Regulation in Mammalian Species. New York, Wiley, pp165–220, 1976.
2. Shrago E, Shug A, Elson C. Regulation of cell metabolism by mitochondrial transport systems. In: Hanson RW, Mehlman MA, eds. Gluconeogenesis: Its Regulation in Mammalian Species. New York, Wiley, pp221–238, 1976.
3. Parrilla R, Ayuso-Parrilla MS. Cellular metabolite distribution and control of gluconeogenesis in perfused isolated rat liver. Pflugers Arch 362:49–54, 1976.
4. Rognstad R, Katz J. Effects of hormones and of ethanol on the fructose 6-P-fructose 1,6 P_2 futile cycle during gluconeogenesis in the liver. Arch Biochem Biophys 177:337–345, 1976.
5. Clark MG, Jarrett IG. Responsiveness to glucagon by isolated rat hepatocytes controlled by the redox state of the cytosolic nicotinamide adenine dinucleotide couple acting on adenosine 3':5' cyclic monophosphate phosphodiesterase. Biochem J 176:805–816, 1978.
6. Feliu JE, Hue L, Hers H. Hormonal control of pyruvate kinase activity and of gluconeogenesis in isolated hepatocytes. Proc Nat Acad Sci USA 73:2762–2766, 1976.
7. Pilkis SF, Claus TH, Johnson RA, Park CR. Hormonal control of cyclic 3':5' AMP levels and gluconeogenesis in isolated hepatocytes from fed rats. J Biol Chem 250:6328–6336, 1975.
8. Chan TM, Freedland RA. Effects of glucagon on gluconeogenesis from lactate and propionate in the perfused rat liver. Proc Soc Exp Biol Med 151:372–375, 1976.
9. Coufalik AH, Monder C. Stimulation of gluconeogenesis by cortisol in fetal rat liver in organ culture. Endocrinology 108:1132–1137, 1981.
10. Menahan LA, Wieland O. The role of thyroid function in the metabolism of perfused rat liver with particular reference to gluconeogenesis. Eur J Biochem 10:188–194, 1969.
11. Singh SP, Snyder AK. Effect of thyrotoxicosis on glu-

gluconeogenesis from alanine in perfused rat liver. Endocrinology 102:182, 1978.

12. Hagenfeldt L, Wennlund A, Felig P, Wahren J. Gluconeogenesis and ketogenesis in hyperthyroidism. Life Sci 26:1851–1856, 1981.
13. Huang MT, Lardy H. Effects of thyroid states on the Cori cycle glucose–alanine cycle and the futile cycling of glucose metabolism in rats. Arch Biochem Biophys 209:41–51, 1981.
14. Muller MJ, Seitz HJ. Dose dependent stimulation of hepatic oxygen consumption and alanine conversion of CO_2 and glucose by 3,5,3′ triiodothyronine in the isolated perfused rat liver of hyperthyroid rats. Life Sci 28:2243–2249, 1981.
15. Meijer AJ, Van Dam K. The metabolic significance of anion transport in mitochondria. Biochem Biophys Acta 346:213–244, 1974.
16. Tobin RB, Berdanier CD, Ecklund RE, DeVore V, Caton C. Mitochondrial shuttle activities in hyperthyroid and normal rats and guinea pigs. J Environ Pathol Toxicol 3:289–305, 1980.
17. Tobin RB, Berdanier CD, Ecklund RE. L-Thyroxine effects upon ATPase activities of several subcellular fractions of liver of rat and guinea pig. J Environ Pathol Toxicol 2:1247–1266, 1979.
18. Ismail-Beigi F, Edelman IS. Time course of the effects of thyroid hormone on respiration and Na^+K^+ATPase activity in rat liver. Proc Soc Exp Biol Med 146:983–988, 1974.
19. Sterling K, Brenner MA, Sakurada T. Rapid effect of thyroid hormone on the mitochondrial pathway in rat liver in vivo. Science 110:340–342, 1980.
20. Sterling K. Thyroid hormone action at the cellular level. N Engl J Med 300:117–123, 173–177, 1979.
21. Hoch FL. Adenine nucleotide translocation in liver mitochondria of hypothyroid rats. Arch Biochem Biophys 178:535–545, 1977.
22. Herd PA. Thyroid hormone-divalent cation interactions. Arch Biochem Biophys 188:220–225, 1978.
23. Harris EJ, Al-Shaikhaly M, Baum H. Stimulation of mitochondrial calcium efflux by thiol-specific reagents and by thyoxine. Biochem J 182:455–464, 1979.
24. Sheare SB, Bronk JR. Effect of thyroxine treatment, in vivo and in vitro, on Ca^{2+} efflux from rat liver mitochondria. FEBS Lett 126:9–12, 1981.
25. Tobin RB, Berdanier CD, Ecklund RE. Effect of L-thyroxine treatment on the levels of various hepatic metabolites in rats. J Environ Pathol Toxicol 2:1223–1234, 1979.
26. Berdanier CD, Shubeck D. Effects of thyroid hormone on mitochondrial activity in lipemic BHE rats. Proc Soc Exp Biol Med 166:348–354, 1981.
27. Berdanier CD. Rat strain differences in gluconeogenesis by isolated hepatocytes. Proc Soc Exp Biol Med 169:74–79, 1982.
28. Cornell NW, Lund P, Krebs HA. Effect of lysine on gluconeogenesis from lactate in rat hepatocytes. Biochem J 162:327–337, 1974.
29. Krebs HA, Cornell NW, Lund P, Hems R. Isolated liver cells as experimental material. In: Alfred Benzon Symposium VI. Copenhagen, Munksgaard, pp726–750, 1974.
30. Stein MW. Glucose. In: Bergmeyer HU, ed. Methods of Enzymatic Analysis. New York, Academic Press, p117, 1963.
31. Williamson DH, Mellanby J. β-Hydroxybutarate and acetoacetate. In: Bergmeyer HU, ed. Methods of Enzymatic Analysis. New York, Academic Press, p459, 1963.
32. Lamprecht W, Trautschold J. ATP. In: Bergmeyer HU, ed. Methods of Enzymatic Analysis. New York, Academic Press, p2101, 1963.
33. Steele RC, Torrie JH. Principles and Procedures of Statistics. New York, McGraw–Hill, p81, 1960.
34. Krebs HA, Freedland RA, Hems R, Stubbs M. Inhibition of gluconeogenesis by ethanol. Biochem J 112:117–124, 1960.
35. Berdanier CD, Tobin RB, DeVore V. Studies on the control of lipogenesis: Strain differences in hepatic metabolism. J Nutr 109:247–260, 1979.
36. Berdanier CD, Tobin RB, DeVore V, Wurdeman R. Studies on the metabolism of glycerol by hyperlipemic and normolipemic rats. Proc Soc Exp Biol Med 157:5–11, 1978.
37. Story DL, O'Donnel JA, Dong FM, Freedland RA. Gluconeogenesis in isolated hepatocytes from fed and 48 hour starved rats. Biochem Biophys Res Commun 73:799–806, 1976.
38. Siess EA, Brocks DG, Wieland OH. Distinctive effects of glucagon on gluconeogenesis and ketogenesis in hepatocytes. Biochem J 172:517–521, 1978.
39. Marshall MW, Knox VA, Trout DL, Durand AMA, Benton DA. Biotin status and lipid metabolism in young inbred rats. Nutr Rep Int 5:201–212, 1972.
40. Lee YP, Takemori AE, Lardy H. Enhanced oxidation of α-glycerophosphate by mitochondria of thyroid fed rats. J Biol Chem 234:3051–3058, 1959.
41. Sokoloff RL, Kaufman S, Campbell PL, Francis CM, Gelboin HV. Thyroxine stimulation of amino acid incorporation into protein. J Biol Chem 238:1432–1437, 1963.
42. Tata JR, Widnell CC. Ribonucleic acid synthesis during the early actions of thyroid hormones. Biochem J 98:604–620, 1966.
43. Soling HD, Kleineke J. Species dependent regulation of hepatic gluconeogenesis in higher animals. In: Hanson RW, Mehlman MA, eds. Gluconeogenesis: Its Regulation in Mammalian Species. New York, Wiley, p369, 1976.
44. Bottger I, Kriegel H, Wieland O. Role of thyroid function in the metabolism of perfused rat liver with particular reference to gluconeogenesis. Eur J Biochem 10:188–194, 1969.

45. Llobera M, Herrera E. Effect of starvation on in vivo gluconeogenesis in hypo and hyperthyroid rats. Endocrinology 106:1628–1633, 1980.
46. Berdanier CD. Rat strain differences in response to meal feeding. Nutr Rep Int 11:517–524, 1975.
47. Otto DA, Ontko JA. Activation of mitochondrial fatty acid oxidation by calcium. J Biol Chem 253:789–799, 1978.
48. Zahlten RN, Kneer NM, Stratman FW, Lardy HA. Influence of ammonium and calcium ions on gluconeogenesis in isolated rat hepatocytes and their response to glucagon and epinephrine. Arch Biochem Biophys 161:528–535, 1974.
9. Kneer NM, Wagner MJ, Lardy HA. Regulation by calcium of hormonal effects on gluconeogenesis. J Biol Chem 254:12160–12168, 1979.
0. Sugano T, Shiota M, Khono H, Shimada M, Oshino N. Effects of calcium ions on the activation of gluconeogenesis by epinephrine in perfused rat liver. J Biochem 87:465–472, 1980.
51. Meijer AJ, Gimpel JA, Deleeuw G, Tischler ME, Tager JM, Williamson JR. Interrelationships between gluconeogenesis and ureogenesis in isolated hepatocytes. J Biol Chem 253:2308–2320, 1978.
52. Berdanier CD. Diet and genetic aspects of maturity-onset lipemia and glycemia. In: Beers RF, Bassett EG, eds. Nutritional Factors: Modulating Effects on Metabolic Processes. Raven Press, New York, pp15–33, 1981.
53. Berdanier CD, Thomson AR, Bouillon D, Wander R. Relevance of hepatic mitochondrial activity to hepatic lipogenesis in genetically different strains of rats. In: Reiser S, ed. Metabolic Effects of Utilizable Dietary Carbohydrates. Marcel Dekker, New York, 1982.

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Vasodilator Responses to Cholinergic and Adrenergic Stimulants in Spontaneously Hypertensive (SHR) and Wistar–Kyoto (WKY) Normotensive Rats (41545)

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Abstract. The cardiovascular effects of isoprenaline, methacholine, and sodium nitroprusside were studied in spontaneously hypertensive (SHR) and in Wistar–Kyoto normotensive (WKY) rats. In conscious rats with chronic indwelling arterial cannulae, methacholine caused a much greater fall in mean arterial blood pressure (MAP) in SHR rats, abolishing the initial 30–40 mm Hg difference in MAP at a dose of 2.5 μg/kg. The hypotensive response to methacholine was accompanied by reflex tachycardia in WKY rats but by a dose-related bradycardia in SHR rats. Isoprenaline increased heart rate and reduced blood pressure to a similar extent in rats of both strains. Administration of sodium nitroprusside resulted in a hypotensive response that was of greater duration in SHR rats. In addition, the reflex tachycardia was more marked and of greater duration in WKY compared to SHR rats. This demonstrates that the baroreflex response to vasodilation is suppressed in SHR rats and that this may contribute to the increased vasodepressor response of these animals to methacholine. However, when baroreflexes but not initial MAP were suppressed with pentobarbital, methacholine caused a similar degree of bradycardia in SHR and WKY rats but a greater vasodepressor response in SHR rats. With isoprenaline, the MAP difference between strains was maintained at all doses up to 200 ng/kg. Although it is not yet clear what is responsible for the increased vascular constriction of SHR rats, our findings suggest that it can be removed by muscarinic receptor activation with methacholine but not by stimulation of β-adrenoceptors.

Spontaneously hypertensive (SHR) rats, originally developed by Okamoto and Aoki (1), are widely regarded as an appropriate animal model for essential hypertension in humans (2, 3). In both SHR rats and in some hypertensive patients, it has been suggested that the development of hypertension may be associated with increased sympathetic tone (4, 5). Such increases in sympathetic nervous system activity could bring about a reduction in the sensitivity of vascular β-adrenoceptors which mediate vasodilation to catecholamines, leaving the vasoconstrictor effects unopposed (6). This suggestion was supported by *in vitro* data which demonstrated that the sensitivity of aortic strip preparations from SHR rats to the relaxant effects of isoprenaline was significantly less than for normotensive controls (7, 8).

The present study was undertaken to compare the effects of isoprenaline, methacholine, and sodium nitroprusside on the cardiovascular system of 8-month-old conscious SHR and Wistar–Kyoto normotensive (WKY) rats. By 8 months of age, SHR rats have sustained elevations in blood pressure as well as several complications of hypertension, including left ventricular hypertrophy, impaired renal function, and structural changes in the vasculature (2, 3). Thus, any differences between strains in their cardiovascular responses to drugs must be viewed as resulting from genetic differences between SHR and WKY rats and from the numerous complications of established hypertension observed for SHR rats. It has been demonstrated that the usual indirect procedure for measuring arterial blood pressure in conscious rats (tail-cuff method) causes increases in plasma catecholamines, particularly in SHR rats, because of the restraining and heating procedures (9). Since these effects would obviously obscure or distort the effects of isoprenaline and methacholine, blood pressure was measured directly by means of a permanent indwelling tail artery cannula while rats remained undisturbed in their home cages. Under such conditions, plasma catecholamines remain low and are similar in SHR and WKY rats (5, 8, 10).

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Methods. Adult male SHR and WKY rats, 8 months of age and weighing 375–450 g, were obtained from Taconic Farms, Germantown, New York, and housed in groups of 4 or 5 for at least one week prior to use. Food and water were available continuously and the vivarium was maintained on a 12-hr light:12-hr dark photoperiod (lights on at 0600 hr). A PE 50 cannula was inserted into the ventral caudal artery of each SHR and WKY rat while under pentobarbital anesthesia (9). In some experiments, a cannula of silastic tubing was also inserted into the left jugular vein, led out at the back of the neck and taped to the spring wire covering the arterial cannula. Rats were then housed singly in plastic cages and the cannulae were flushed twice daily with 0.5 ml of 0.9% saline containing 500 U/ml of sodium heparin. For experiments on anesthetized rats, animals were injected with pentobarbital (40–50 mg/kg, ip) and PE 50 cannulae were inserted into the ventral caudal arteries. Drug studies were then performed using this acute preparation. An additional dose of pentobarbital (10 mg/kg) was given approximately one hour after the first to maintain a stable level of anesthesia.

Blood pressure was measured by connecting the end of the arterial cannula directly to a Statham pressure transducer (P23AC) with tracings made on a Grass polygraph. Heart rate was measured with a Grass tachometer that was triggered by fluctuations in arterial blood pressure. Using this technique, direct measures of mean arterial blood pressure and heart rate can be obtained from conscious rats that are undisturbed in their home cages. Drugs were injected into the arterial cannula by using a three-way stopcock or were injected directly into the venous cannula. All drug injections were given in a volume of 0.2 ml and were flushed in with 0.2 ml of 0.9% saline. Three to five doses of either isoprenaline or methacholine were injected intraarterially twice into each rat in ascending order of magnitude at intervals of not less than 5 min to avoid tachyphylaxis. Only one drug was administered to each of eight pairs of SHR and WKY rats. Sodium nitroprusside (2 μg/kg) was injected intravenously into conscious SHR and WKY rats. Control injections of 0.2 ml of saline were also performed and were not observed to have significant effects on MAP or heart rate. The maximum changes in mean arterial pressure and heart rate were recorded for a given dose of each drug. In addition, the duration of action of isoprenaline and sodium nitroprusside was estimated for each animal by measuring the time from drug injection until the mean arterial pressure and heart rate returned to preinjection values.

Drugs were obtained from the following sources: l-isoprenaline sulfate from Sterling Winthrop, Rensselaer, New York, methacholine chloride from Aldrich Chemical Company, Milwaukee, Wisconsin, and sodium nitroprusside from Roche Laboratories, Nutley, New Jersey. Solutions of isoprenaline (including ascorbic acid, 10 μg/ml, as an antioxidant), methacholine, and sodium nitroprusside were prepared freshly in 0.9% saline before each experiment and stored on ice until use. All doses are expressed as nanograms or micrograms per kilogram of the salt. Student's *t* test was used to compare the mean values for MAP and heart rate of SHR and WKY rats.

Results. The average resting MAP ± SE of all conscious SHR rats in this study was 153 ± 5 mm Hg compared to a value of 116 ± 3 mm Hg for conscious WKY rats ($P < 0.01$). The mean heart rate of SHR rats (330 ± 6 beats/min) was also higher than that of WKY rats (306 ± 7 beats/min) ($P < 0.05$).

Isoprenaline increased heart rate by similar amounts and for similar durations in conscious SHR and WKY rats at each of three doses (Fig. 1 and Table 1). MAP was also reduced in SHR and WKY rats by the same

TABLE 1 DURATION OF CHANGES IN MEAN ARTERIAL PRESSURE (MAP in mm Hg) AND HEART RATE (HR, beats/min) IN CONSCIOUS SHR AND WKY RATS FOLLOWING ADMINISTRATION OF ISOPRENALINE

Dose of isoprenaline (ng/kg)	MAP		HR	
	SHR	WKY	SHR	WKY

Values are means ± SE.
* $P < 0.05$.
** $P < 0.01$.

amount at each dose level so that the initial strain difference in MAP of 30 mm Hg was maintained (Fig. 2). However, the duration of the fall in MAP was significantly longer (P's < 0.005) for SHR rats at each of the three doses of isoprenaline (Table 1).

Methacholine caused a pronounced reflex tachycardia in WKY rats while in SHR rats only a dose-related bradycardia was observed (Fig. 1). In addition, methacholine caused a much greater fall in MAP in SHR rats. Thus, at a dose of 2.5–5 μg/kg of methacholine, the MAP reached the same level in the two strains of rats in spite of an initial difference between strains of 42 mm Hg (Fig. 2).

Pentobarbital anesthesia did not signifi- cantly lower the resting MAP of either s of rats, nor did it raise the heart rate of rats. However, the heart rate of WKY rat increased significantly following pentob tal anesthesia from 306 ± 7 to 335 ± 9 b min (P < 0.05). The increase in heart ra duced by isoprenaline was similar in thetized rats compared to conscious rats was of similar magnitude in the two st at each of five dose levels. On the other h pentobarbital anesthesia abolished the tachycardia of WKY rats induced by m choline, converting it to a dose-related dycardia similar to that seen in SHR rats 3). Isoprenaline lowered the MAP of thetized SHR and WKY rats by sir

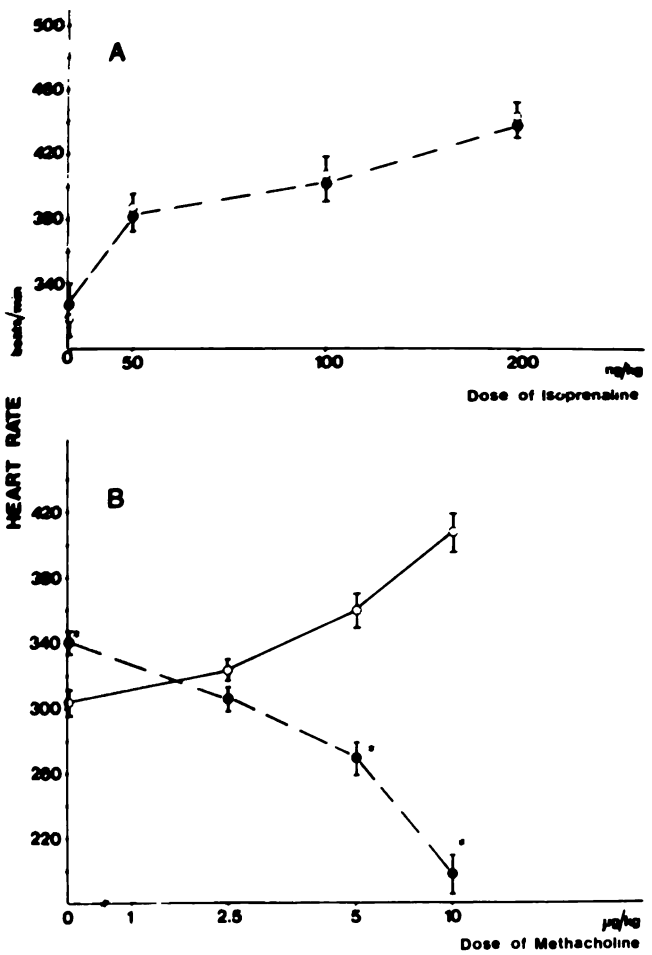

FIG. 1. Effects of isoprenaline (A) and methacholine (B) on heart rate in conscious SHR and WKY rat ○, WKY rats; ●, SHR rats. * denotes significant from WKY rats (P < 0.05).

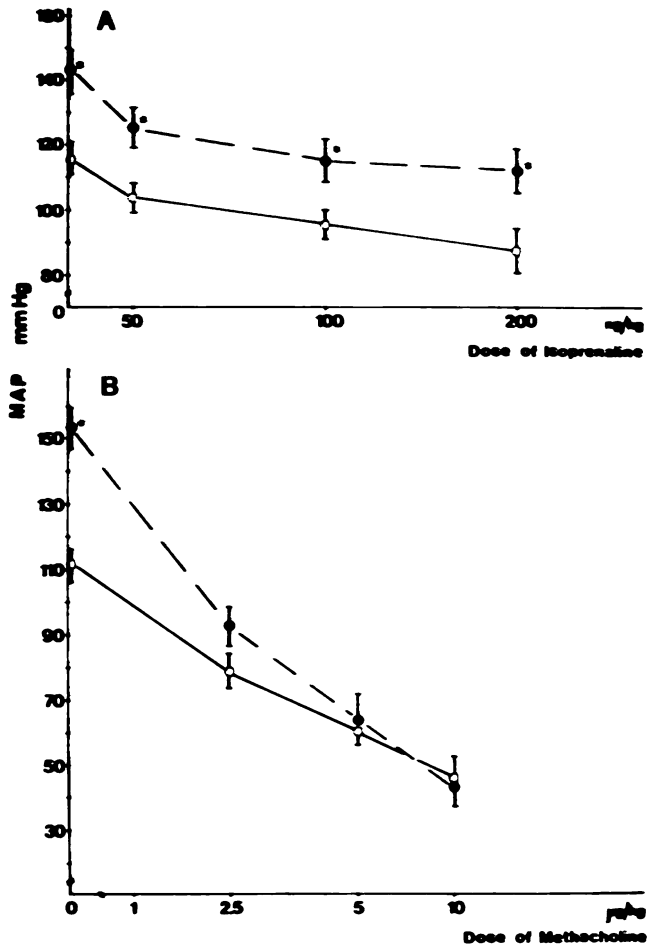

FIG. 2. Effects of isoprenaline (A) and methacholine (B) on MAP in conscious SHR and WKY rats. O, WKY rats; ●, SHR rats. * denotes significant difference from WKY rats ($P < 0.05$).

amounts and for similar durations, so that at each dose level the difference between strains in MAP remained between 26 and 35 mm Hg (Fig. 4 and Table II). In anesthetized rats, isoprenaline caused a significant reduction of MAP at lower doses when compared to conscious rats of the same strain. For example, in SHR rats a dose of 50 ng/kg of isoprenaline produced a fall in MAP of 12 ± 1 mm Hg in conscious animals and 29 ± 3 mm Hg in anesthetized animals ($P < 0.01$). Similarly, in WKY rats a dose of 50 ng/kg of isoprenaline produced a fall in MAP of 16 ± 2 mm Hg in conscious animals and 30 ± 4 mm Hg in anesthetized animals ($P < 0.05$) (compare Figs. 2 and 4).

Anesthetized SHR and WKY rats were also more sensitive to the vasodepressor effects of methacholine than were conscious rats, especially at doses of 1 μg/kg and less. In addition, methacholine had a more marked hypotensive effect in SHR than WKY rats. For example, a dose of 1 μg/kg of methacholine produced a fall in MAP of 83 ± 5 mm Hg in anesthesized SHR rats and 41 ± 6 mm Hg in anesthesized WKY rats ($P < 0.01$). Thus, the initial strain difference in MAP of 43 mm Hg in anesthesized rats was abolished by a dose of 1 μg/kg of methacholine (Fig. 4).

Intravenous injection of sodium nitroprusside (2 μg/kg) into conscious rats resulted in a pronounced fall in MAP of greater intensity

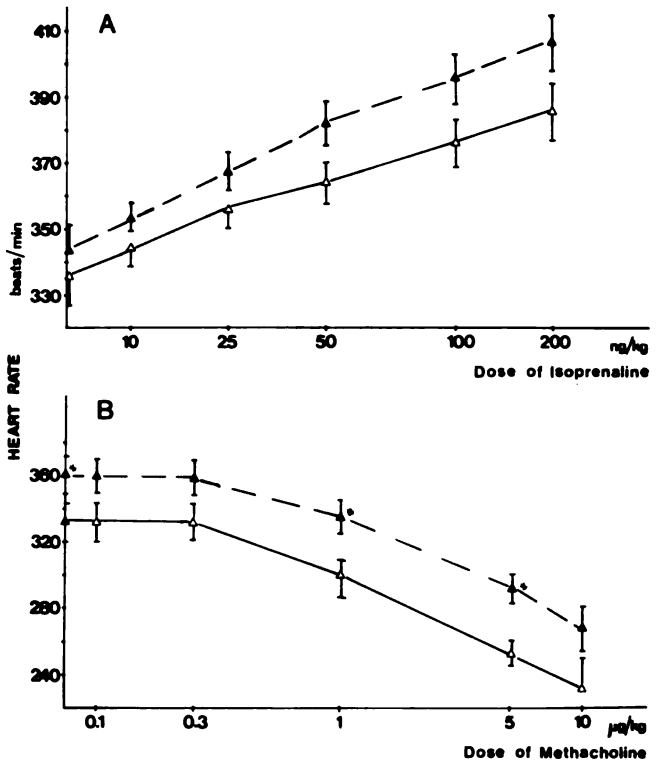

FIG. 3. Effects of isoprenaline (A) and methacholine (B) on heart rate in pentobarbital-anesthetized SHR and WKY rats. △, WKY rats; ▲, SHR rats. * denotes significant difference from WKY rats ($P < 0.05$).

but of much shorter duration than after the highest dose of isoprenaline (Table III). The duration of the hypotensive effect of sodium nitroprusside was approximately 44% greater in SHR rats ($P < 0.01$). Although the final heart rate reached after this dose of nitroprusside was not different in the two groups of rats the increase in heart rate of 130 ± 11 beats/min was more marked ($P < 0.05$) and of greater duration ($P < 0.01$) in WKY than SHR rats.

Discussion. The direct effects of isoprenaline on the cardiovascular system which result from activation of β-adrenoceptors are: (a) stimulation of the SA node to produce tachycardia, (b) stimulation of cardiac muscle to increase contractile force, and (c) dilation of peripheral blood vessels to produce hypotension. Methacholine, acting on muscarinic cholinergic receptors, causes vasodilation and has negative inotropic and chronotropic effects on the heart. In contrast, nitroprusside produces vasodilation by a direct action on vascular smooth muscle. These actions also result in a number of indirect effects, which include activation of baroreceptor reflex mechanisms as a result of a lowering of pressure in the aortic arch and carotid sinus. This reflex response stimulates sympathetic nervous system activity, inhibits vagal influences on the heart and inhibits sympathetic cholinergic vasodilator fibers in skeletal muscle to cause tachycardia and vasoconstriction (11). These indirect actions of the drugs would be expected to hasten the termination of the hypotensive response and restore blood pressure to its control level.

The present findings in conscious SHR and WKY rats demonstrate the considerable influence of baroreceptor reflex activity on the vasodilator responses to isoprenaline, methacholine, and nitroprusside. In addition, this activity was found to be impaired in SHR rats. Previous studies using vasoconstrictor agents have also shown that baroreceptor function is deficient in hypertensive rats (12-

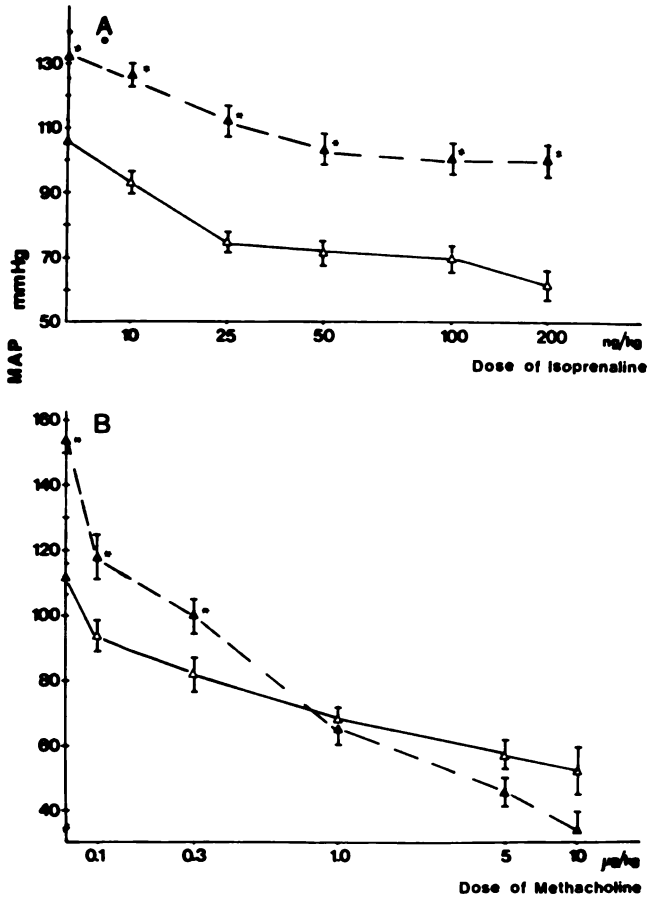

FIG. 4. Effects of isoprenaline (A) and methacholine (B) on MAP in pentobarbital-anesthetized SHR and WKY rats. △, WKY rats; ▲, SHR rats. * denotes significant difference from WKY rats ($P < 0.05$).

TABLE II. DURATION OF CHANGES IN MEAN ARTERIAL PRESSURE (MAP, mm Hg) AND HEART RATE (HR, beats/min) OF PENTOBARBITAL-ANESTHETIZED SHR AND WKY RATS FOLLOWING ADMINISTRATION OF ISOPRENALINE[a]

Dose of isoprenaline (ng/kg)	Duration of change (sec)			
	MAP		HR	
	SHR	WKY	SHR	WKY
10	26 ± 5	54 ± 14	36 ± 13	66 ± 27
25	85 ± 6	100 ± 15	88 ± 15	144 ± 37
50	119 ± 11	121 ± 3	174 ± 31	203 ± 39
100	143 ± 8	153 ± 18	224 ± 16	216 ± 40
200	205 ± 24	205 ± 48	281 ± 40	305 ± 54

[a] Values are means ± SEM for groups of four to seven rats per strain.

14). Thus, while the vasodepressor response to methacholine resulted in dose-related tachycardia in WKY rats, only marked bradycardia occurred in SHR rats. There was no significant difference in the tachycardia induced by isoprenaline in the two rat strains.

The hypotensive effect of methacholine (5 and 10 μg/kg) in SHR rats was almost double that in WKY rats. The greater hypotensive response of SHR rats to methacholine is similar to the observation that hypertensive humans may react to methacholine with a significant fall in blood pressure (15). In contrast, isoprenaline and nitroprusside caused similar decreases in MAP in the two strains of rats. It is possible that the exaggerated depressor response of SHR rats to methacholine

TABLE III. EFFECTS OF SODIUM NITROPRUSSIDE (2 μg/kg, iv) ON MEAN ARTERIAL PRESSURE (mm Hg) AND HEART RATE (beats/min) IN CONSCIOUS SHR AND WKY RATS[a]

	Mean arterial pressure		Heart rate	
	SHR	WKY	SHR	WKY
Before drug	145 ± 5**	110 ± 3	326 ± 8	307 ± 14
Change after drug	−53 ± 5	−42 ± 5	97 ± 8*	130 ± 11
Duration of response (sec)	64 ± 6**	44 ± 2	62 ± 4**	92 ± 10

[a] Values are means ± SEM for seven rats per strain.
* $P < 0.05$.
** $P < 0.01$ compared to WKY rats.

may have resulted from a lack of reflex sympathetic stimulation and vagal inhibition and not from any difference in the action of this drug on blood vessels.

Our findings with nitroprusside support the view that SHR rats have a diminished baroreceptor reflex response to vasodilation. Thus, for an equal drop in mean arterial pressure, WKY rats had a greater increase in heart rate and blood pressure returned to baseline levels more quickly than for SHR rats. To our knowledge, this is one of the few studies on baroreceptor reflexes in SHR rats that have employed vasodilators. In a recent report (16), prehypertensive and hypertensive Dahl genetically salt-sensitive rats were shown to have an impaired baroreceptor reflex response to vasoconstriction with phenylephrine but not to vasodilation with nitroglycerin. Taken together, these results indicate that the disturbances in baroreceptor reflexes may differ in the various animal models of hypertension.

Although baroreceptor reflexes can be abolished by a combination of nicotinic and muscarinic receptor blockade of ganglionic transmission (10) or by spinalization, the resulting low blood pressure would make it impossible to study quantitatively the vasodepressor responses to isoprenaline or methacholine. However, pentobarbital anesthesia has been shown to suppress cardiovascular reflexes and this was accomplished without a significant decrease in blood pressure (11, 18), as was also illustrated in the present study. The inhibitory effect of pentobarbital on parasympathetic influences (11) was also seen in the significant tachycardia it induced in WKY rats. The suppression of baroreceptor reflex activity by pentobarbital was further demonstrated in the present study by the findings that methacholine failed to cause tachycardia in anesthetized WKY rats but only a dose-related bradycardia of similar magnitude to that seen in SHR rats. Thus, in pentobarbital-anesthetized rats, the direct effects of isoprenaline and methacholine predominate.

Although we did not measure cardiac output in our rats, it is likely that the effect of isoprenaline on the heart was similar in SHR and WKY rats, as heart rate was increased by the same amount in both strains at each dose level following induction of anesthesia. Similarly, methacholine caused the same degree of dose-related bradycardia in anesthetized SHR and WKY rats. This suggests that any differences in the vasodepressor responses of SHR and WKY rats to isoprenaline or methacholine probably do not result from a differential effect of the drugs on cardiac output.

Methacholine (1 μg/kg) was able to bring the blood pressure to the same level (68 mm Hg) in SHR and WKY rats in spite of the considerable strain difference in initial MAP. This suggests that SHR rats are more sensitive to the direct effects of methacholine on blood vessels, which, together with some baroreflex sensitivity impairment, results in the greater overall vasodepressor response to this agent. In contrast, several doses of isoprenaline and a single dose of nitroprusside failed to abolish the difference in blood pressure between the two strains, thereby demonstrating a differential sensitivity of blood vessels of SHR to different vasodilator agents.

Previous studies have shown that vascular relaxation mechanisms appear to be impaired in SHR rats (8). Vasodilation is believed to result from the sequestering of calcium by microsomes via an ATP-dependent mechanism (19). Recent research suggests that this mech-

anism is deficient in microsomes from blood vessels of SHR rats (20). The results from the present study suggest that stimulation of muscarinic cholinergic receptors can overcome any initial difference in the contractility of the blood vessels of SHR and WKY rats. This reveling action does not appear to be shared by the β-adrenoceptor agonist, isoprenaline, suggesting that β-adrenoceptor activation and cyclic AMP production cannot abolish the increased vascular constriction of SHR rats. It would be of interest to determine if methacholine can normalize the calcium sequestering activity and calcium mobilizing ability of microsomes in blood vessels of SHR rats such that the initial strain differences in calcium movement are minimized.

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1. Okamoto K, Aoki K. Development of a strain of spontaneously hypertensive rats. Japan Circ J 27:282–293, 1963.
2. Trippodo NC, Frohlich ED. Similarities of genetic (spontaneous) hypertension: Man and rat. Circ Res 48:309–319, 1981.
3. Yamori Y. Pathogenesis of spontaneous hypertension as a model for essential hypertension. Japan Circ J 41:259–266, 1977.
4. DeChamplain J, Farley L, Cousineau D, van Ameringen MR. Circulating catecholamines in human and experimental hypertension. Circ Res 38:109–114, 1976.
5. McCarty R, Kopin IJ. Alterations in plasma catecholamines and behavior during acute stress in spontaneously hypertensive and Wistar-Kyoto normotensive rats. Life Sci 22:997–1005, 1978.
6. Amer MS. Mechanisms of action of β-blockers in hypertension. Biochem Pharmacol 26:171–175, 1977.
7. Amer MS, Bomoll AW, Perhach JL, Ferguson HC, McKinney GR. Alterations in cyclic nucleotide metabolism in the hearts and blood vessels of hypertensive rats. Proc Nat Acad Sci USA 71:4930–4934, 1974.
8. Cohen ML, Berkowitz BA. Decreased vascular relaxation in hypertension. J Pharmacol Exp Ther 196:396–406, 1976.
9. Chiueh CC, Kopin IJ. Hyperresponsivity of hypertensive SHR rats to indirect measurement of blood pressure. Amer J Physiol 234:H690–H695, 1978.
10. McCarty R, Kvetnansky R, Lake CR, Thoa NB, Kopin IJ. Sympathoadrenal activity of SHR and WKY rats during recovery from forced immobilization. Physiol Behav 21:567–572, 1978.
11. Kircheim HR. Systemic arterial baroreceptor reflexes. Physiol Rev 56:100–176, 1976.
12. Nosaka S, Okamoto K. Modified characteristics of the aortic baroreceptor activities in the spontaneously hypertensive rat. Japan Circ J 34:685–693, 1970.
13. Nosaka S, Want SC. Carotid sinus baroreceptor functions in the spontaneously hypertensive rat. Amer J Physiol 222:1079–1085, 1972.
14. Pfeffer MA, Frohlich ED. Hemodynamics of spontaneously hypertensive rats. I Effects of pressure elevations. Amer J Physiol 224:1066–1071, 1973.
15. Goodman LS, Gilman AG. The Pharmacological Basis of Therapeutics, 5th ed. New York, Macmillan, 1975.
16. Gordon FJ, Matsuguchi H, Mark AL. Abnormal baroreflex control of heart rate in prehypertensive and hypertensive Dahl genetically salt-sensitive rats. Hypertension 3 (Suppl I):I135–I141, 1981.
17. Steinberg M, Hilton JG. Effects of chloroisondamine and atropine on cardiovascular responses to stimulation of the carotid sinus. Tex Rep Biol Med 24:643–649, 1966.
18. Page IH, McCubbin JW. Autonomic regulation of arterial pressure responses. Arch Int Pharmacodyn 157:152–165, 1965.
19. Moore L, Hurwitz L, Davenport GR, Landon EJ. Energy-dependent calcium uptake activity of microsomes from the aorta of normal and hypertensive rats. Biochem Biophys Acta 413:432–443, 1975.
20. Mushlin PS, Rama Sustry BV, Boerth RC, Surber MJ, Landon EJ. Dithiothreitol-induced alterations of blood pressure, muscular reactivity and aortic microsomal calcium uptake in spontaneously hypertensive rats. J Pharmacol Exp Ther 207:331–339, 1979.

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PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 172, 202–206 (1983)

Identification of the Virus-Specific Proteins of Respiratory Syncytial Virus Temperature-Sensitive Mutants by Immunoprecipitation (41548)

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Abstract. The proteins of Long strain RSV and three temperature-sensitive (ts) mutants of the A2 strain were compared by pulse labeling virus-infected cells with [^{35}S]methionine and [^{3}H]glucosamine followed by analysis of the cell lysates by polyacrylamide gel electrophoresis. At the permissive temperature (30°) proteins ranging in molecular weight from 24,000 to 50,000 (VP24, VP27, VP33, VP44) could be identified. Immunoprecipitation of viral lysates by immune rabbit serum demonstrated antigenic similarity with VP27, VP44, VP50, and VP67 in all ts mutants and Long strain RSV. [^{3}H]Glucosamine labeling demonstrated glycoproteins of 90,000 (GP90) and 50,000 (GP50) in Long strain and GP90 in the ts mutants.

Respiratory syncytial virus (RSV) is the major cause of serious respiratory disease in infants. An RNA virus, RSV is a 120- to 300-nm-diameter particle (1) with spikes protruding from its envelope, one of which is felt to be a fusion protein accounting for the characteristic syncytial cytopathic effect. Initial attempts at vaccination using a formalin-inactivated RSV vaccine were unsuccessful, with vaccine recipients developing a more severe natural RSV illness than controls (2). It has been speculated that altered immunological reactivity to the wild viral infection, perhaps caused by formalin denaturation of the fusion protein in the vaccine, may have contributed to these adverse effects (3). Failure of inactivated viral vaccines led to the development and testing of cold-adapted temperature-sensitive (ts) mutants of RSV. Using 5-fluorouridine as a mutagen, Gharpure produced multiple temperature-sensitive (ts) mutants of the A2 (Australian) strain of RSV (4). These ts mutants have been well characterized with regard to their growth characteristics and genetic stability at the permissive (30°) and restrictive (37°) temperatures, and have been categorized into at least seven complimentation groups (A through G) (5–8). To date the ts-1 and ts-2 mutants have been evaluated as possible vaccine strains (9, 10).

In order to be an effective vaccine, an attenuated ts mutant should replicate in the vaccine recipient and present an antigenic challenge similar to the wild-type virus. This would require ts mutant synthesis of structural proteins which are antigenically similar to the parent strain. We therefore compared the presence of the proteins of ts-1, ts-2, and ts-7 grown in tissue culture at the permissive and restrictive temperature to the Long strain of RSV grown at 30° and 37°, respectively. In addition, those proteins capable of immunoprecipitation with rabbit hyperimmune serum were compared using radiolabeling of viral proteins and analysis of polyacrylamide gel electrophoresis (PAGE), a technique we have previously utilized to demonstrate Long strain viral proteins (11).

Materials and Methods. *Cells and virus.* Hep-2 cell lines were grown in Eagle's minimum essential medium (MEM) containing 5% fetal calf serum (FCS). Stock RSV Long strain and ts-1, ts-2, and ts-7 (all obtained from R. Chanock) were grown at 30° (except RSV, grown at 37°) in Hep-2 cells to titers of 2–6 $\times 10^5$ plaque-forming units (PFU)/ml, and frozen at −70° until use.

Antibody to RSV. Antibody to RSV was produced in New Zealand white rabbits by injection of purified Long strain virus grown in bovine embryonic kidney cells as previously described in detail (11). This antibody has no reactivity against Hep-2 cells.

Radiolabeling of RSV. Hep-2 cells, grown in six-well Costar plates, were infected with each strain at the following multiplicities of infection (m.o.i.): Long strain RSV (m.o.i.

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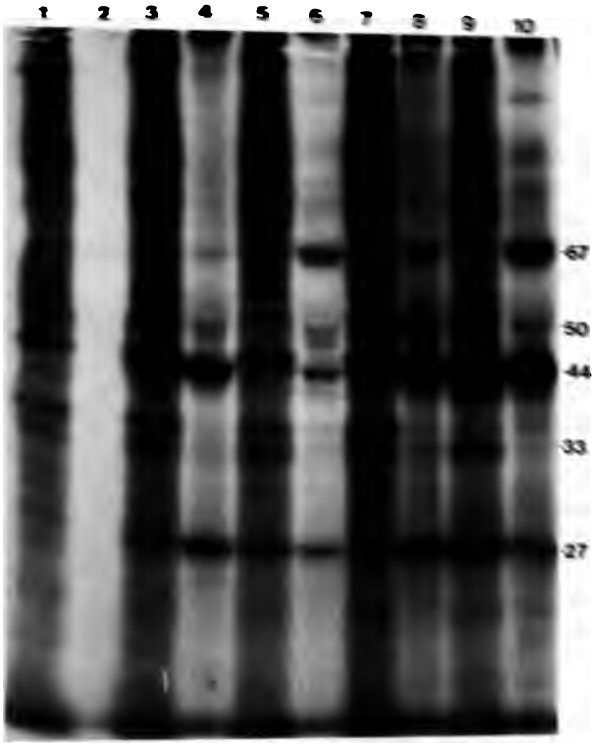
1 (m.o.i. = 0.2), ts-2 (m.o.i. = 0.2), (m.o.i. = 0.1). After a 90-min ab- period at room temperature the me- is aspirated and replaced with 3 ml MEM containing 5% agammaglob- al calf serum, and incubated in 5% 30° or 37°. When early CPE was de- the medium was aspirated and re- with methionine-free MEM without staining [^{35}S]methionine at a concen- of 10 μCi/ml (Amersham Corp.). Be- and 24 hr later the medium was as- The cell sheet was lysed with 0.5 ml uffer (0.01 *M* Tris–HCl, pH 7.4, 0.15 L 1% sodium desoxycholate, 1% Tri- 00, 0.1% sodium dodecyl sulfate) con- 1 m*M* phenylmethylsulfonyl fluoride . This lysate was then analyzed by

ar labeling techniques were used to radiolabel with [^{3}H]glucosamine, except that the labeling medium was MEM with 30% of the usual dextrose concentration supple- mented with [^{3}H]glucosamine, 10–50 μCi/ml (Amersham Corp.).

Immunoprecipitation. Immunoprecipita- tion and polyacrylamide gel electrophoresis were carried out by previously described methods (11).

Results. *Identification of viral proteins at the permissive temperature 30°.* Growth of Long strain RSV and the temperature-sensi- tive mutants ts-1, ts-2, and ts-7 in HEp-2 cells at 30° resulted in the synthesis of four pre- sumed viral proteins which were not seen in uninfected cells (see Figs. 1 and 2). Three of these proteins had molecular weight (MW) of 44,000, 33,000, and 27,000 daltons and were seen with all RSV strains. The fourth protein of 24,000-dalton molecular weight was seen



G. 1. Proteins of Long strain RSV and the ts mutants grown at the permissive temperature (30°). Virus grown at 30° for 48 hr, then pulse labeled with [^{35}S]methionine, lysed in RIPA buffer, immunopre- ted with rabbit anti-RSV sera, electrophoresed on a 12% polyacrylamide gel, and fluorographed. ples applied to the gel were as follows: HEp-2 cell lysate (1), immunoprecipitate of HEp-2 (2), Long n RSV lysate (3), immunoprecipitate of Long strain (4), ts-1 lysate (5), immunoprecipitate of ts-1 (6), lysate (7), immunoprecipitate of ts-2 (8), ts-7 lysate (9), and immunoprecipitate of ts-7 (10).

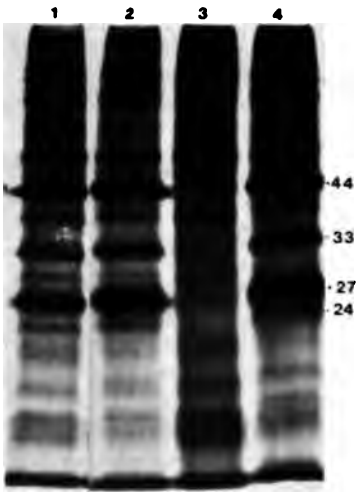


FIG. 2. Proteins of Long strain RSV and the ts mutants grown at the permissive temperature (30°), as in Fig. 1, but run on a 10% polyacrylamide gel. Samples applied to the gel are ts-1 lysates (1), ts-7 lysates (2), HEp-2 lysates (3), and Long strain RSV lysates (4). This gel resolves a viral peptide with MW 24,000 daltons.

only in the Long strain, ts-1, and ts-7; this latter polypeptide is best resolved on a 10% polyacrylamide gel as shown in Fig. 2.

Immunoprecipitation of the cell culture lysates with specific anti-RSV rabbit immune serum revealed viral-specific polypeptides of MW 67,000, 50,000, 44,000, and 27,000 daltons for each of the ts mutants and Long strain (Fig. 1).

Identification of viral-specific proteins at the restrictive temperature (37°). At the restrictive temperature ts-2 was the only temperature-sensitive strain which readily labeled with [^{35}S]methionine, demonstrating polypeptides of 50,000, 44,000, 33,000, and 27,000 daltons (figure not shown). The proteins of ts-1 and ts-7 were poorly discerned at 37°. In an attempt to radiolabel ts-1 and ts-7 at 37°, shift-up experiments were done. In these experiments the virus was initially incubated at 30° for 48 hr. Cultures were then incubated at 37° and [^{35}S]methionine was added at various time points after shift-up to 37°. However, even after shift-up, only trace amounts of proteins of MW 50,000, 44,000, and 27,000 daltons could be identified for ts-1 and ts-7 (Fig. 3). Long strain and ts-2 proteins, by contrast, were well labeled during shift-up experiments demonstrating polypeptides with MW of 44,000, 33,000, 27,000 and 24,000 daltons. Additionally with both Long strain and ts-2 a protein of MW 10,000 daltons was also noted in these experiments (Fig. 3).

Identification of viral glycoproteins at permissive temperature. Previous work had shown that Long strain RSV contained two virally specified glycopeptides of 50,000- and 90,000-dalton molecular weight; however, the 90,000-dalton polypeptide was labeled only with [^{3}H]glucosamine but not with [^{35}S]-methionine (11). After [^{3}H]glucosamine labeling of Long strain RSV, ts-1, ts-2, and ts-7 at 30°, a 90,000-dalton polypeptide was seen with all strains (Fig. 4). However, at 37° only in the Long strain RSV cultures was the

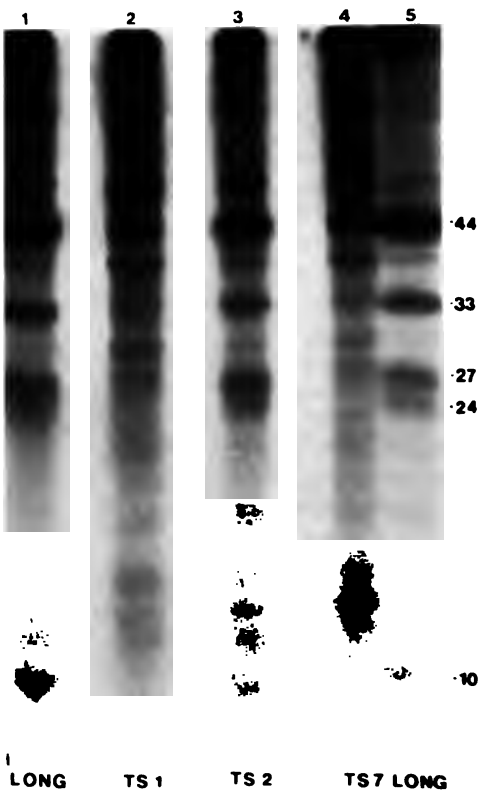
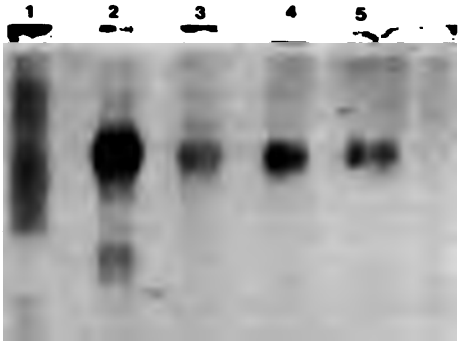


FIG. 3. Protein of Long strain and ts mutants after step-up to the restrictive temperature (37°). In this experiment virus was incubated at 30° for 48 hr, then stepped up to 37° and pulse labeled after 3 hr at 37°. Long strain (lanes 1 and 5) and ts2 (lane 3) demonstrate proteins MW 44K, 33K, 27K, 24K, and 10K. ts1 (lane 2) and ts7 (lane 4) are poorly labeled.



3. 4. [^{3}H]Glucosamine-labeled protein of Long strain and the ts mutants grown at the permissive temure (30°). Virus was grown at 30° in HEp-2 cells for , then labeled with [^{3}H]glucosamine, lysed with RIPA r, electrophoresed on a polyacrylamide slab gel and ıgraphed. Samples applied to gel are cell lysates of 2 (1), Long strain RSV (2), ts-1 (3), ts-2 (4), and ts-

00-dalton glycopeptide seen (figure not vn). In addition, at 30° a 50,000-dalton oprotein was seen in the Long strain RSV not with the ts mutants.

iscussion. These labeling experiments carried out with three ts mutants and Long strain of RSV. The ts mutants, ts-2, and ts-7 have previously been catized into complimentation groups A, B, C, respectively. Prior to the labeling exments plate complimentation studies were ied out to confirm the separate compliıtation groups of each ts mutant. In adın the inability of ts-2 to form syncytia at 30° and 37° was documented.

he labeling studies presented here demrate that at the permissive temperature), the temperature-sensitive mutants and g strain produce proteins of similar size the exception of the 24,000-dalton profor ts-2. However, in the step-up labeling riment this protein was seen for ts-2. The ificance of this is not clear. Immunopreıation of these proteins with hyperimmune rabbit sera demonstrated similar proteins consistently. Using [^{3}H] glucosamine as the label, a 90K MW glycoprotein, presumably a surface viral glycoprotein, was noted in all ts mutants and Long strain at the permissive temperature, whereas the 50K MW glycoprotein could only be demonstrated for Long strain. However, using [^{35}S]methionine as the label, all ts mutants demonstrated a 50K MW protein, suggesting that the absence of labeling by [^{3}H]glucosamine may have been a technical problem related to the relatively lighter labeling of ts mutants with [^{3}H]-glucosamine, as seen in Fig. 4.

These data would suggest that at the permissive temperature of 30°, which approximates human nasal temperature, these ts mutants produce analogous proteins with similar antigenic reactivity as the Long strain of RSV as measured by their ability to be immunoprecipitated by anti-RSV antibody. This has potentially important implications in the use of ts mutants as vaccine strains, especially in view of the adverse results with the inactivated RSV vaccine. It has been speculated that the more severe natural RSV infection seen following vaccination with formalin-inactivated virus may have been due to the inactivation of the antigenicity of the fusion protein by formalin. This could result in the failure of vaccines to produce antibodies to the fusion protein, and thus alter their immunological interaction with natural viral infection. This would be most relevant to the nonsyncytial forming mutant, ts-2, which presumably produces a nonfunctional fusion protein. Thus far, it has not been proven which of RS viral proteins is the fusion protein.

In addition these studies give additional support to the identifications of the proteins of RSV. Labeling with [^{35}S]methionine of the ts mutants demonstrated proteins of 67,000, 50,000, 44,000, 33,000, 27,000, 24,000, and 10,000 daltons, while labeling with [^{3}H]glucosamine demonstrates a glycoprotein of 90,000 and 50,000 daltons.

Recently, Pringle has demonstrated similar protein synthesis by these ts mutants; however, the immunologic reactivity with anti-RSV antibody was not documented by specific immunoprecipitation (12). Several differences between their studies and ours are evident. They did not demonstrate a protein

of MW 67,000 daltons; we could only demonstrate this protein by specific immunoprecipitation, a technique they did not use. In addition we did not identify a protein of MW 200,000. This may be due to the poor resolving ability of 10–12% polyacrylamide gels at that molecular weight.

1. Bloth BA, Espmartz A, Norrby E, Gard S. The ultrastructure of respiratory syncytial virus. Arch Gesamte Virusforsch 13:582–586, 1963.
2. Kapikian AZ, Mitchell RH, Chanock RM, Shvedoff RA, Stewart CE. An epidemiologic study of altered clinical reactivity to respiratory syncytial (RS) virus infection in children previously vaccinated with an inactivated RS virus vaccine. Amer J Epidemiol 89:405–421, 1969.
3. Choppin PW, Scheid A. The role of viral glycoproteins in adsorption, penetration, and pathogenicity of viruses. Rev Infect Dis 2:40–61, 1980.
4. Gharpure MA, Wright PF, Chanock RM. Temperature-sensitive mutants of respiratory syncytial virus. J Virol 3:414–421, 1969.
5. Giminez HB, Pringle CR. Seven complementation groups of respiratory syncytial virus temperature-sensitive mutants. J Virol 27:459–464, 1978.
6. Wright PF, Gharpure MA, Hodes DS, Chanock RM. Genetic studies of respiratory syncytial virus temperature-sensitive mutants. Arch Gesamte Virusforsch 41:238–247, 1973.
7. Belshe RB, Richardson LS, Schnitzer TJ, Prevar DA, Camargo E, Chanock RM. Further characterization of the complementation group B temperature-sensitive mutant of respiratory syncytial virus. J Virol 24:8–12, 1977.
8. Schnitzer TJ, Richardson LS, Chanock RM. Growth and genetic stability of the ts-1 mutant of respiratory syncytial virus at restrictive temperatures. J Virol 17:431–438, 1976.
9. Kim HW, Arrobio JO, Brandt CD, Wright P, Hodes D, Chanock RM, Parrott RH. Safety and antigenicity of temperature sensitive (ts) mutant respiratory syncytial virus (RSV) in infants and children. Pediatrics 52:56–63, 1973.
10. Belshe RB, Richardson LS, London WT, Sly DL, Camargo E, Prevar DA, Chanock RM. Evaluation of five temperature-sensitive mutants of respiratory syncytial virus in primates. J Med Virol 3:101–110, 1978.
11. Bernstein JM, Hruska JF. Respiratory syncytial virus proteins: Identification by immunoprecipitation. J Virol 38:278–285, 1981.
12. Pringle CR, Shirodaria PV, Gimenez HB, Levine S. Antigen and polypeptide synthesis by temperature-sensitive mutants of respiratory syncytial virus. J Gen Virol 54:173–183, 1981.

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Permeability of Polyethylene Glycol in Remnant Small Bowel after Massive Intestinal Resection (41547)

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Abstract. Studies were designed to determine if permeability of adapted (remnant) small bowel mucosa to polyethylene glycol (PEG) was altered after major intestinal resection. Rats underwent 50% small bowel resection with preservation of duodenum and terminal ileum. Sham-operated animals served as controls. Two and four weeks later we cannulated the portal vein and measured mucosal permeability to luminal [^{3}H]PEG and [^{14}C]PEG in isotonic Ringer solution in remnant proximal or distal in situ closed intestinal loops. A lumen-to-portal blood gradient of at least 1000/1 persisted throughout the one-hour experimental period in both resected and sham-operated animals. Thus the adapted remnant intestinal mucosa was highly impermeable to luminal radiotracer PEG. In separate experiments 2 and 4 weeks after 70% small bowel resection or sham operation, *in vivo* segments of proximal and distal small intestinal were perfused through the lumen for one hour with hypertonic (800 mOsm) mannitol or NaCl solution containing [^{3}H]PEG. There was equal and almost total recovery of [^{3}H]PEG at the end of the experimental period in resected and control animals. The combined data of all experiments indicate that radiotracer PEG may be confidently used as a luminal water phase marker in transport studies of remnant bowel following intestinal resection.

Since its introduction in 1947 (1) polyethylene glycol (PEG), molecular weight 4000, has been used in both man and experimental animals as a nonabsorbable water phase luminal volume marker in a wide variety of *in vivo* studies of intestinal transport. It has become the accepted standard marker though chemical methods of assay have remained laborious (2-5). The availability of radiolabeled PEG has served to simplify its assay (5) and recent work has shown that radiotracer PEG without the presence of stable carrier PEG in the luminal solution suffices as a water phase marker (3, 6). Intestinal resection results in both morphologic and functional adaptive changes in remnant bowel (7, 8). The rate of mucosal cell turnover in remaining intestine increases and there may be a consequent functional immaturity (9-12). Increased permeability of immature neonatal mucosa to macromolecules has been amply documented (13, 14). The permeability to PEG of remnant mucosa after intestinal resection may be altered. If so, PEG would be unsuitable for use as an indicator for luminal water movement.

Two types of experiments were performed. In rats 2 and 4 weeks after major small bowel resection mucosal permeability was examined by instilling isotonic Ringer solution containing either [^{3}H]PEG or [^{14}C]PEG into ligated loops of remnant proximal or distal small bowel. Radiotracer concentration of PEG was then measured sequentially in portal blood. In other experiments total recovery of luminal [^{3}H]PEG was measured after 1 hr *in vivo* recirculation of hypertonic solution through the lumen of segments of remnant small intestine. The results show that radiolabeled PEG, molecular weight 4000, is a valid reference substrate for luminal water movement in the adapted postresected rat intestine.

Materials and Methods. Sprague-Dawley-derived male albino rats were used in all experiments. The rats were allowed free access to tap water and commercial rat chow (Wayne Lab Blox, Allied Mills, Libertyville, Ill.) and were randomly assigned for either small bowel resection or sham operation. Food but not water was withheld overnight before surgery which was carried out under intraperitoneal (ip) pentobarbital anesthesia (4 mg/100 g body weight). Rats subjected to intestinal resection underwent laparotomy and either 50 or 70%

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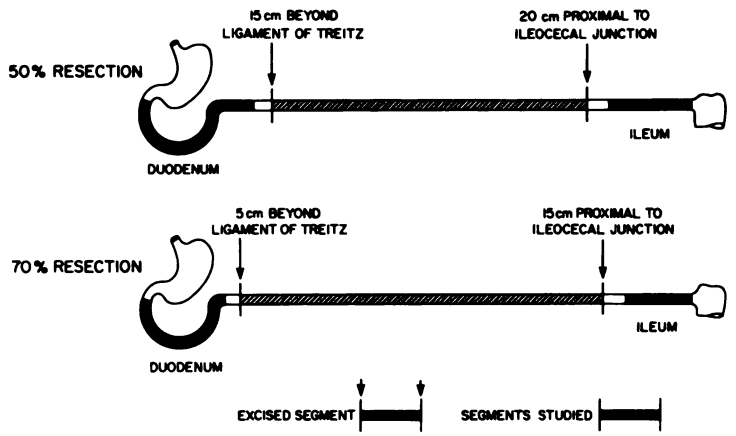

FIG. 1. Diagram showing locations of the excised segment in the two types of resected rats and the segments studied 2 or 4 weeks later. Rats with 50% small bowel resection were utilized for the closed loop experiments and one *in situ* loop was constructed in each animal. Rats with 70% small bowel resection were used for *in vivo* luminal perfusion and both proximal and distal segments were studied simultaneously in these animals. Sham-operated rats (small bowel transected at mid-small intestine followed by end-to-end reanastomosis) served as controls for each type of resection and similar segments were studied in these animals.

small bowel resection (Fig. 1). Rats from the sham-operated group underwent laparotomy and mid-small intestinal transection without removal of any intestinal tissues. In each instance continuity of the intestinal lumen was reestablished by end-to-end anastomosis. Operative details have been described (8). Either 2 or 4 weeks after surgery (ranges 13–15 days and 26–30 days) animals were anesthetized with ip urethane (0.2 g/100 g body weight) and the following experiments were performed.

Closed loop experiments. In rats with 50% intestinal resection or sham operation the portal vein was cannulated as previously described (15). An *in situ* closed intestinal loop about 20 cm long was constructed from either proximal or distal small bowel. The proximal loop began at the duodenopyloric junction and ended 10–12 cm distal to the ligament of Treitz (Fig. 1). In these animals the common bile duct was also ligated close to its entrance into the duodenum. The distal loop utilized the terminal 18–20 cm of ileum (Fig. 1). These loops will be referred to as duodenum and ileum. The loops were filled with 3.5 ml isotonic Ringer solution (16) containing about 30,000 cpm/0.1 ml tracer [^{3}H]PEG or [^{14}C]PEG (NET-405, Lot 1282-46; NET-473, Lot 1291-285, New England Nuclear Corp., Boston, Mass.). Just prior to and for five 10-min intervals after filling the intestinal loop, 0.5-ml aliquots of portal vein blood were collected (15), allowed to clot, and after centrifugation the supernatant serum was harvested for assay of radioactivity. At the end of each experiment the intestinal loop was emptied, excised, measured for length, and weighed. Aliquots, 0.1 ml of portal vein sera as well as luminal solution before and at the end of an experiment were measured for ^{3}H or ^{14}C radioactivity by standard liquid scintillation methodology. A vial containing only scintillator was used to measure background radiation with all estimations of portal vein sera radioactivity. Background radiation was subtracted from the measured radioactivity of all portal vein serum samples.

Luminal recirculation. Following anesthesia and laparotomy, the common bile duct was ligated in all rats subjected to 70% intestinal resection (Fig. 1) or sham operation. Inflow and outflow cannulas were tied into the proximal 10–12 cm and into the distal 10–12 cm of the small intestine of each animal (Fig. 1). *In vivo* luminal perfusion at 2 ml/min was then carried out. The method closely followed our prior description (8) except that the luminal perfusate was either hypertonic (800 mOsm) mannitol or hypertonic (800 mOsm) NaCl with approximately 20,000 cpm/ml

:G (NET-405, Lot 1244-104). The per- was recirculated for 1 hr from duodenal al reservoirs each containing 25 ml so- At the end of the perfusion period the its were emptied by flushing with air. men of each segment was then washed ing 0.9% saline through it at a rate of nin. The effluents from each segment ollected separately in three 10-min al- Pilot studies showed that the last 10- nse contained only minimal radioac- All fluid volumes were determined by ng. Radioactivity was measured by rd methodology in 1-ml aliquots of the ite before and after luminal perfusion 1-ml aliquots of the saline rinsing so- . Percentage recovery of radiotracer as calculated as follows: The total ra- vity present in the reservoir of each seg- it the beginning of luminal perfusion nl × initial volume) was the denomi- of the fraction; the numerator was the adioactivity in the reservoir after 1 hr lation (cpm/ml × final volume) plus nbined radioactivities for the three sa- ashes (cpm/ml × rinse volume).

experimental data are given as mean Statistical evaluation is based on Stu- *t* test and *P* values of less than 0.05 aken to indicate statistically significant nces.

alts. *Closed loop experiments.* Table I animal and intestinal data for control and resected rats 2 and 4 weeks after entry into the study. Twice as many animals were utilized in the 4 weeks group because we studied intestinal loops filled with Ringer solution and [^{14}C]PEG as well as loops filled with Ringer solution and [^{3}H]PEG. Mean body weights of each group of animals at entry into the study were identical. Two or four weeks after surgery mean body weights of the resected animals were numerically less than controls but the differences were not significant. Lengths of the loops filled with Ringer solution containing tracer PEG also did not differ. However, the weights of intestine, normalized for length, were significantly increased in both duodenum and ileum of resected rats at both study times. Table II shows concentrations of tracer PEG in the luminal solution both prior to instillation into the loops and at the conclusion of experiments 1 hr later. In duodenum, luminal tracer PEG concentrations increased significantly during all experiments, the increase in resected animals being significantly more than in the sham-operated control groups. In ileum the increase in concentration of luminal tracer PEG occurred only in resected animals. The data suggest that significant absorption of water (and presumably electrolyte) occurred in the duodenum of all animals and that there were adaptive changes of water absorption in both duodenum and ileum after resection. Although methodologies differed, the data are

TABLE I. INTESTINAL LOOPS: CONTROL AND RESECTED RATS

	2 Weeks[a]		4 Weeks[a]	
	Sham	Resected	Sham	Resected
ight (g)[b]				
	162 ± 3 (12)	172 ± 6 (12)	168 ± 3 (24)	169 ± 3 (24)
	231 ± 7 (12)	226 ± 7 (12)	285 ± 5 (24)	278 ± 5 (24)
l loops				
enum				
gth (cm)	19.9 ± 1.0 (6)	21.4 ± 3.1 (6)	19.1 ± 0.5 (12)	18.8 ± 0.5 (12)
t weight (mg/cm)[c]	72.6 ± 5.0 (6)	98.9 ± 10[d] (6)	73.9 ± 2.5 (12)	100.6 ± 2.6[d] (12)
gth (cm)	20.4 ± 1.6 (6)	18.2 ± 0.5 (6)	19.8 ± 0.4 (12)	19.0 ± 0.5 (12)
t weight (mg/cm)[c]	68.1 ± 2.0 (6)	104.2 ± 4.9[d] (6)	68.3 ± 3.0 (12)	101.5 ± 2.1[d] (12)

nals were studied 2 or 4 weeks after 50% small bowel resection or sham operation; values are mean ± SE; in parentheses are the number of rats.
ing weights immediately prior to induction of anesthesia.
thickness intestinal tissue.
ificantly different ($P < 0.05$) from corresponding data of sham-operated animals.

TABLE II. INTESTINAL LOOPS: RADIOACTIVITY OF LUMINAL SOLUTION AND PORTAL VEIN BLOOD

	2 Weeks [^{3}H]PEG[a]				4 Weeks [^{3}H]PEG[a]				4 Weeks [^{14}C]PEG[a]			
	Duodenum		Ileum		Duodenum		Ileum		Duodenum		Ileum	
	Sham	Resected	Sham	Resected	Sham	Resected	Sham	Resected	Sham	Resected	Sham	Resected
	Intestinal solution (cpm/0.1 ml)											
Initial	30,800 ± 200	33,600 ± 400	31,000 ± 100	30,900 ± 300	30,200 ± 200	30,100 ± 600	29,900 ± 400	28,700 ± 400	32,900 ± 500	32,700 ± 100	32,300 ± 300	32,500 ± 400
After 1 hr	37,000 ± 200[b]	51,700 ± 3100[b,c]	32,000 ± 600	41,000 ± 2800[b,c]	35,000 ± 400[b]	54,400 ± 100[b,c]	33,200 ± 600	42,200 ± 2400[b,c]	38,600 ± 500[b]	51,900 ± 3200[b,c]	33,600 ± 600	45,700 ± 2200[b,c]
Time (min)	Portal vein, serum (cpm/0.1 ml)[d]											
0	10 ± 6	10 ± 6	5 ± 2	7 ± 2	3 ± 1	12 ± 4	7 ± 1	10 ± 4	12 ± 4	16 ± 5	11 ± 3	12 ± 3
10	40 ± 4	28 ± 3	24 ± 5	27 ± 2	28 ± 2	31 ± 2	25 ± 3	36 ± 8	25 ± 4	28 ± 7	20 ± 4	17 ± 4
20	39 ± 7	25 ± 4	23 ± 3	26 ± 3	29 ± 2	33 ± 2	24 ± 2	31 ± 4	22 ± 2	26 ± 3	22 ± 3	22 ± 3
30	36 ± 3	32 ± 3	24 ± 4	28 ± 2	30 ± 2	34 ± 2	28 ± 3	31 ± 4	21 ± 3	21 ± 5	25 ± 4	18 ± 3
40	39 ± 2	28 ± 4	22 ± 4	26 ± 3	29 ± 1	38 ± 2	27 ± 2	31 ± 5	20 ± 2	20 ± 4	25 ± 3	16 ± 4
50	39 ± 6	33 ± 4	22 ± 5	27 ± 3	29 ± 1	40 ± 2	26 ± 2	31 ± 4	19 ± 2	21 ± 3	24 ± 3	20 ± 4
60	36 ± 4	31 ± 4	25 ± 5	27 ± 2	31 ± 1	37 ± 2	26 ± 1	30 ± 5	21 ± 3	21 ± 4	24 ± 2	23 ± 4

[a] Intestinal loops in animals studied 2 weeks after entry were filled with Ringer solution containing [^{3}H]PEG, loops in animals studied 4 weeks after entry were filled with Ringer solution containing either [^{3}H]PEG or [^{14}C]PEG; all values are mean ± SE of six observations.

[b] Significantly different ($P < 0.05$) than initial luminal tracer PEG concentration.

[c] Significantly different from corresponding data of sham-operated animals.

[d] Background radioactivity has been subtracted.

TABLE III. *In Vivo* LUMINAL PERFUSIONS WITH 800 mOsm MANNITOL

	2 Weeks[a]		4 Weeks[a]	
	Sham	Resected	Sham	Resected
Body weight (g)[b]				
Initial	156 ± 4	157 ± 4	161 ± 6	163 ± 5
Final	241 ± 9	226 ± 8	312 ± 10	294 ± 12
Intestinal segments				
Duodenum				
Mucosa, wet weight (mg/cm)	29.7 ± 2.0	37.7 ± 2.7[d]	28.7 ± 2.1	39.9 ± 2.0[d]
Water, secretion (ml/cm in 1 hr)[c]	0.150 ± 0.025	0.114 ± 0.022	0.142 ± 0.030	0.118 ± 0.014
[^{3}H]PEG, % recovery	96.1 ± 1.8	98.4 ± 1.7	98.8 ± 1.1	100.4 ± 1.1
Ileum				
Mucosa, wet weight (mg/cm)	27.3 ± 3.6	41.1 ± 3.1[d]	26.4 ± 1.8	44.7 ± 4.7[d]
Water, secretion (ml/cm in 1 hr)	0.058 ± 0.014	0.094 ± 0.017[d]	0.066 ± 0.012	0.101 ± 0.014[d]
[^{3}H]PEG, % recovery	99.9 ± 1.0	98.5 ± 1.3	98.0 ± 1.2	98.3 ± 0.7

[a] Animals were studied 2 or 4 weeks after 70% small bowel resection or sham operation; values are mean ± SE of 10 observations.
[b] Fasting weights immediately prior to induction of anesthesia.
[c] Determined from initial and final weights of perfusate reservoirs.
[d] Significantly different ($P < 0.05$) from corresponding data of sham-operated animals.

consistent with adaptations of transport activities in the postresected remnant small intestine demonstrated for several luminal substrates (7, 8, 12). Table II also shows radioactivities of portal vein sera obtained before instilling Ringer solution with tracer PEG into the intestinal loop (0 min and at 10-min intervals thereafter). In all sham and resected animals the radioactivity of portal vein blood remained very low for the whole hour compared to radioactivity in the intestinal lumen. A lumen-to-portal blood gradient of at least 1000/1 was maintained throughout this period. There were no further sequential increases of portal blood radioactivities between 10 and 60 min after the initial slight increase in the first 10 min. The data clearly indicate a very high degree of mucosal impermeability to both [^{3}H]PEG and [^{14}C]PEG.

Luminal recirculations. Table III shows data from experiments where 800 mOsm mannitol solution containing [^{3}H]PEG was recirculated for 1 hr. Body weights, both initial and final, follow the same trends as in the prior experiments. Similarly the weight of intestinal mucosa, normalized for segment lengths, also increased significantly in duodenum and ileum of resected rats at the two study times.

In the sham-operated animals at both study times net water secretion into the luminal segments was significantly greater in duodenum than ileum. In ileum after resection the increase in water secretion approximated mucosal growth. However, in duodenum where mucosal mass also increased, net water secretion did not differ from the sham-operated groups. The postresected duodenal mucosa thus exhibits a protective capacity against hyperosmolar luminal challenge not shown by remnant ileum. In spite of these different responses the mean recovery of [^{3}H]PEG at the end of the experimental period was greater than 96% in all groups of animals. Similar experiments with luminal perfusate containing 800 mOsm NaCl with [^{3}H]PEG yielded almost identical [^{3}H]PEG recoveries (data not shown). These data indicate that in the postresected remnant intestine loss of luminal [^{3}H]PEG either by transport through the mucosa or adsorption onto mucosa and tubing was minimal.

Discussion. The basic assumption underlying the use of water phase luminal volume markers in studies of intestinal transport is that concentration changes of the marker accurately reflect luminal water movement. Ideally this requires total mucosal impermeability with complete recovery of the marker from the luminal solution at the end of the experimental period. In normal intestine Carbowax 4000 or polyethylene glycol, molecular weight 4000, closely approaches this ideal (1–6).

Intestinal resection results in considerable hyperplasia of intestinal tissues. Mucosal hyperplasia occurs rapidly after enterectomy (9). It has usually been considered to have reached its maximum extent by 4 weeks after resection (10) and to persist for several months at least (17). In this study, maximum morphologic adaptation occurred 2 weeks after resection (Tables II and III), in close agreement with a prior report of maximum changes 12 days after enterectomy (11). The mucosa may exhibit a functional immaturity (9–12). The permeability of this mucosa to PEG has not been systematically examined.

Experiments with *in situ* intestinal loops clearly demonstrate that only very minimal increases in radioactivity occurred in portal blood following luminal instillation of Ringer solution containing tracer PEG (Table II). The differences between resected rats and sham-operated controls were inconsequential. The small increase in portal blood radioactivity seen in the first 10 min after instillation of substrate into the lumen was most likely the consequence of minor mucosal damage at the time of manipulation of the loops during instillations of luminal solution and tying of the final ligature. Over the next 50 min there were no further increases in portal blood radioactivities and a lumen-to-portal vein blood gradient of at least 1000/1 persisted. We deliberately used a luminal radioactivity concentration that was 15 times larger than necessary for transport studies (such as those utilized in subsequent luminal perfusions with hypertonic solutions).

Because of the very minimal mucosal permeability to both [^{3}H]PEG and [^{14}C]PEG in the closed loop experiments we decided to study radiotracer PEG recoveries after *in vivo* intestinal perfusions in animals subjected to a more extensive (70%) intestinal resection. We only examined [^{3}H]PEG recovery because there was no difference in mucosal permeability to either of the tracers (Table II). Additional stress was placed upon mucosal integrity by using strongly hypertonic luminal solutions instead of isotonic solutions. Significant increases in small intestinal mucosal permeability have been demonstrated for metabolically inert oligosaccharides during hyperosmolar luminal stimuli (18). The technique of luminal perfusion has been well established for studies of *in vivo* intestinal transport (3, 8). Following 1 hr luminal recirculation more than 96% [^{3}H]PEG was recovered from duodenum and ileum of both resected and sham-operated rats (Table III). Miller and Schedl (3) have previously reported at least 96% recovery of [^{14}C]PEG from the intestine of normal rats after 1 hr luminal perfusion with an isotonic solution.

In conclusion, the data clearly show that following enterectomy the adapted mucosa of remnant small bowel presents a formidable barrier to the absorption of radiolabeled luminal PEG and there is almost total recovery of tracer PEG after *in vivo* luminal perfusion. Therefore the material may be confidently used as a water phase marker for estimating luminal water movement in the postresected rat intestine.

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1. Schaffer DC, Critchfield FH. Solid polyethylene glycols (carbowax compounds): Quantitative determinations in biological materials. Anal Chem 19:32–34, 1947.
2. Jacobson ED, Bondy DC, Broitman SA, Fordtran JS. Validity of polyethylene glycol in estimating intestinal water volume. Gastroenterology 44:761–767, 1963.
3. Miller DL, Schedl HP. Total recovery studies of non absorption indicators in the rat small intestine. Gastroenterology 58:40–46, 1970.
4. Buxton TB, Crockett JK, Moore III WL, Prissing JP. Protein precipitation by acetone for the analysis of polyethylene glycol in intestinal perfusion fluid. Gastroenterology 76:820–824, 1979.
5. Wingate DL, Sandbery JR, Phillips SF. A comparison of stable and ^{14}C labelled polyethylene glycol as volume indicators in the human jejunum. Gut 13:812–815, 1972.
6. Helman CA, Barbezat GO. Comparison of ^{14}C labelled polyethylene glycol (PEG) with carrier PEG and ^{14}C-PEG alone as a volume indicator in the human jejunum. Gut 19:155–156, 1978.
7. Urban E, Weser E. Intestinal adaptation to bowel resection. In: Stollerman GH, ed. Advances in Internal Medicine. Chicago, Year Book Med Pub, Vol 26:pp265–291, 1980.

8. Urban E, Pena M. In vivo calcium transport by rat small intestine after massive bowel resection. Amer J Physiol 226:1304–1308, 1974.
9. Obertop HS, Nundy S, Malamud D, Malt RA. Onset of cell proliferation in the shortened gut. Rapid hyperplasia after jejunal resection. Gastroenterology 72:267–270, 1977.
10. Williamson RCN, Bauer FLR, Ross JS, Malt RA. Proximal enterectomy stimulates distal hyperplasia more than bypass or pancreaticobiliary diverson. Gastroenterology 74:16–23 1978.
11. Hanson WR, Osborne JW, Sharp JG. Compensation by the residual intestine after intestinal resection in the rat. Influence of post operative time interval. Gastroenterology 72:701–705, 1977.
12. Weser E, Hernandez MH. Studies of small bowel adaptation after intestinal resection in the rat. Gastroenterology 60:69–75, 1971.
13. Abrahamson DR, Powers A, Rodewald R. Intestinal absorption of immune complexes by neonatal rats: A route of antigen transfer from mother to young. Science 206:567–569, 1979.
14. Waksman A, Hupert P, Cremel G, Rendon A, Burgun C. Translocation of proteins through biological membranes. A critical review. Biochim Biophys Acta 604:249–296, 1980.
15. Urban E, Zingery AA. A simple method of cannulating the portal vein and obtaining multiple blood samples in the rat. Experientia 37:1036–1037, 1981.
16. Peterson SC, Goldner AM, Curran PF. Glycine transport in rabbit ileum. Amer J Physiol 219:1027–1032, 1970.
17. Weser E, Tawil T. Epithelial cell loss in remaining intestine after small bowel resection in the rat. Gastroenterology 71:412–415, 1976.
18. Wheeler PG, Menzie IS, Creamer B. Effect of hyperosmolar stimuli and coeliac disease on the permeability of the human gastrointestinal tract. Clin Sci Mol Med 54:495–501, 1978.

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PGE_2 and Angiogenesis[1] (41548)

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Abstract. The angiogenic capability of PGE_2 was tested by implanting pellets of an ethylene vinyl acetate slow release polymer containing PGE_2 on the chorioallantoic membrane of 8-day-old chicken embryos. Elvax pellets releasing approximately 0.2, 2.0, or 20 ng/day PGE_2 were found to induce neovascular responses. In contrast, pellets releasing 2.0 or 20 ng/day of either PGA_2, PGF_2, or TXB_2 did not appear to be angiogenic when compared with PGE_2. These release rates of PGE_2 are similar to those reported for a variety of tumors, activated macrophages, inflammatory exudates, and rheumatoid synovia, suggesting that PGE_2 may be a key factor in various neovascular reactions.

New blood vessel formation, angiogenesis, is essential for the successful growth of solid tumors (1, 2). Angiogenesis can be induced not only by tumor cells, but also by allogeneic lymphocytes (3, 4) and by activated macrophages (5). In fact, angiogenesis-inducing factors have been obtained from a large number of tissue sources (6), suggesting that the induction of blood vessels may be critical for a number of biological processes ranging from wound healing to cancer growth, and including vascular reactions associated with inflammation and autoimmune pathology.

The idea that prostaglandins of the E series might play a significant role in many of the angiogenesis-inducing systems has been proposed (6, 7), although experimental data in support of the concept have been quite limited (8). Reports of the association of PGE_2 with tumors (10), activated macrophages (11–13), and inflammatory exudates (14) lend credence to this idea and have prompted us to carry out experiments to examine whether prostaglandin E_2 can induce angiogenesis.

Materials and Methods. The assay system we have employed is the assessment of vascular response on the chlorioallantoic membrane (CAM) of chicken embryos grown in petri dish embryo culture (15). In brief, embryos were explanted from 3-day eggs into 100 × 20-mm plastic tissue culture dishes (Falcon). To provide adequate humidity, each dish was placed in a larger (150 × 25-mm) plastic dish (Falcon) and incubated at 37°. On Day 8, implantation was performed by making an incision in the CAM with a 23-gauge needle and placing the implant over the hole. With sufficient care, bleeding was minimal. This method has the advantage of permitting the monitoring of the neovascular reactions including sequential photography and measurement of the blood vessels surrounding the angiogenesis-stimulating implant.

We used Elvax-40 (ethylene vinyl acetate copolymer, DuPont Chemical Co.) as a slow-release compound into which test samples of prostaglandin (Sigma Chemical Co.) could be introduced (16). The method used was a modification of that used by Rhine *et al.* (16). Elvax was washed at least 50 times in 2.5% ethanol at 37° (at least 3 hr per wash) before use. Washed Elvax was dissolved in methylene chloride (10% w/v) at 37° in siliconized glass tubes. The appropriate prostaglandin, in an ethanol carrier, was added and the mixture was vortexed and poured into glass molds on dry ice. The solidified mixture was dried at −20° for 2 days, and for a further 2 days under mild vacuum at room temperature. This technique resulted in Elvax slabs of consistent dimensions which could be cut into uniform implants.

Disc-shaped pellets, 2 mm diameter, each containing approximately 1 mg of polymer were used as implants on the CAM of 8-day-old chicken embryos. Controls for uni-

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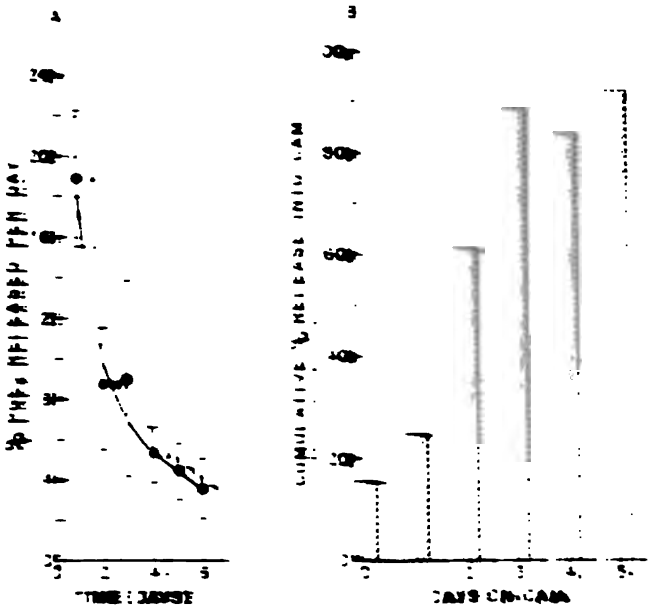

FIG. . Release of [3H]PGE2 from Elvax pellets. (A) Percentage remaining per day. (B) Cumulative percentage released from the CAM. Calculated from the amount of [3H]PGE2 remaining in pellets after implantation on the CAM for various periods of time (22).

ity and release kinetics were carried out using [3H]PGE2 prostaglandin E2 [11,12-3H(N)], New England Nuclear. Pellets containing [3H]PGE2 were placed individually in glass scintillation vials containing 1 ml saline. At 24-hr intervals, the pellets were transferred to fresh scintillation vials.

After 6 days, the experiment was terminated and the yolks were freed and the eyes were removed and enucleated. The release of PGE2 into the saline was calculated from the amount of PGE2 remaining in pellets after implantation on the CAM for various periods of time. Elvax pellets were prepared containing PGE2 or other cyclooxygenase products (PGE1, PGA2, PGF2α, thromboxane B2, and 6-keto-PGF1α). Control pellets consisted of Elvax alone. In order to insure a more even release of PGE2, pellets were soaked in saline for 24 hr before use.

For each experiment, 8-day-old embryos were divided into groups of six, each group receiving only one type of pellet. Two pellets were implanted on each CAM, resulting in 12 implants per group. All embryo cultures were subsequently coded to prevent potential bias [illegible] each pellet was carefully dried to remove all traces of [illegible] solvent in a vacuum desiccator. The weighing method used was a modification of that described by Folkman [illegible]. Pellets containing a wide range of PGE2 [illegible] negative, [illegible] weakly positive, and [illegible] strongly positive. At the end of the experimental period, the embryos were examined with a stereomicroscope. Angiogenesis (CAM) was evaluated in each group by dividing the number of [illegible] score for all pellets by the total number of [illegible] possible score.

For statistical analysis, the data were [illegible] treated as a one-way analysis of variance [illegible] described by the Newman-Keuls [illegible] was [illegible] between groups.

Results. As shown in Fig. 1A, [3H]PGE2 was released gradually from pellets over a 6-day period. Release rates for [illegible] during these three days were found to be [illegible] comparable from [illegible] and were close to the published release rates of Elvax [illegible] (16) for release of [illegible] steroids although [illegible] can be seen in Fig. 1A the initial release was rapid, but a steady slow rate was obtained after the first day and for this reason all pellets used for testing angiogenesis were [illegible]

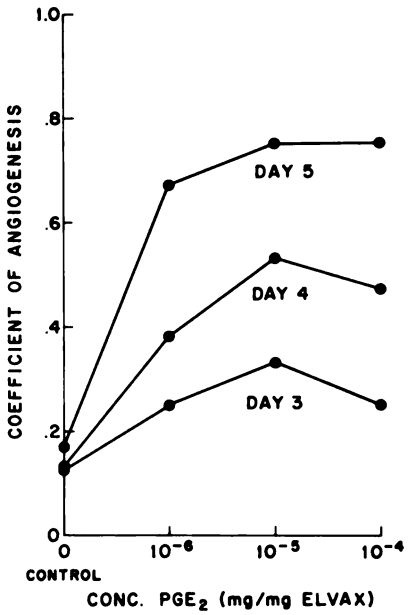

FIG. 2. Coefficient of angiogenesis (COA) calculated for Elvax pellets containing 10^{-6}, 10^{-5}, or 10^{-4} PGE$_2$. COA calculated 3, 4, and 5 days after implantation of the pellets on the CAM of 8-day-old chicken embryos. Values shown were obtained from the median of four experiments (21).

saline for 24 hr before being placed on the CAM for assessment of angiogenesis-inducing activity. The release rates of PGE$_2$ into the CAM during a 5-day period is shown in Fig. 1B.

The response of the CAM vasculature to the presence of PGE$_2$-containing pellets involved two distinct phenomena: (1) the formation of a network of dilated capillaries occurred within 48 hr and declined after 96 hr; and (2) the appearance of bending, looping, and sprouting of blood vessels, with a clear orientation toward the implanted pellet. This reaction was seen beginning on the third day and became increasingly marked over the next several days.

Pellets containing 10^{-4}, 10^{-5}, or 10^{-6} mg PGE$_2$ releasing approximately 0.2, 2.0, or 20 ng/day into the CAM, were tested for their angiogenesis-inducing capacity. The median values of four experiments are shown in Fig. 2. By Day 5, the COA values for all three concentrations of PGE$_2$ were significantly higher ($P < 0.025$) than controls. PGE$_1$ gave results similar to those seen with PGE$_2$ (data not shown).

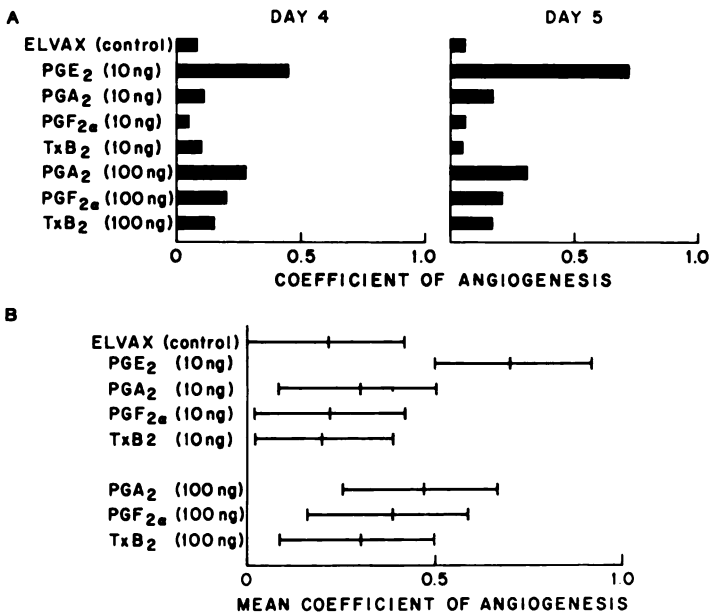

FIG. 3. Comparison of the angiogenesis capacity of PGE$_2$ with that of other prostaglandins. (A) Coefficient of angiogenesis (COA) calculated for Elvax pellets containing 10^{-6} mg PGE$_2$ compared to pellets containing 10^{-5} or 10^{-6} mg PGA$_2$, PGF$_2$, or TxB$_2$. COA calculated 4 and 5 days after implantation of the pellets on the CAM of 8-day-old chicken embryos. (B) Mean COA per egg on Day 5 ± one pooled standard deviation (95% confidence interval).

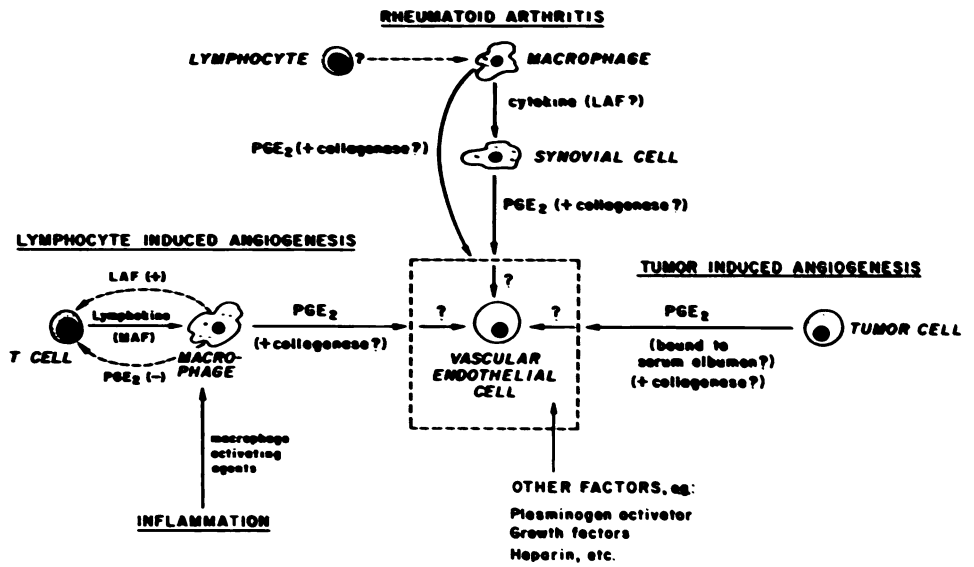

FIG. 4. Possible points of PGE involvement in angiogenesis (see text for discussion).

As a further control for specificity, the angiogenic capability of pellets containing 10^{-4} or 10^{-5} mg PGA_2 (the oxidation product of PGE_2), $PGF_{2\alpha}$, or TxB_2 were compared with pellets containing 10^{-5} mg PGE_2. The results of a typical experiment are shown in Fig. 3. Only PGE_2 is clearly angiogenic.

Discussion. The fact that PGE_2 can be angiogenic in our test system at release rates of as little as 0.2 ng/day induced us to reexamine data on neovascularization induced in a variety of other experimental or clinical situations to determine if the levels of PGE_2 are consistent with its being involved in angiogenesis. Indeed, levels of PGE_2 at or above those used by us have been reported for a variety of tumors (10), for activated macrophages (11–13), and for inflammatory exudates (14), all of which have been associated with angiogenesis. Neovascularization is also found associated with a proliferating vasculature in the synovium, and it is of interest not only that both cultured rheumatoid synovial fragments and adherent cells derived from such fragments produce PGE_2 at high rates (17), but that PGE_2 production can be further enhanced by supernatants from activated macrophages (18).

A major group of neovascular reactions appears to be initiated by antigen-stimulated lymphocytes (lymphocyte-induced angiogenesis, LIA) (6), and is seen in graft-versus-host reactions, autoimmune reactions, and delayed hypersensitivity. Although lymphocytes themselves produce little or no PGE, it may be significant that LIA almost definitely involves release of soluble factors (lymphokines) (3, 4, 19, 20) and there is ample evidence that macrophages activated by lymphokines produce high levels of PGE_2 (11–13).

Biochemical studies of angiogenesis-inducing factors have pointed to two classes of factors: low-molecular-weight ones (200–450 daltons), and larger, nondialyzable ones, some of which may actually involve a small active component attached to a larger carrier molecule (6). Whether the active molecule associated with these factors will prove to be PGE_2 is not clear, but there is a strong possibility that molecules of the prostaglandin–leukotriene family may be involved. Figure 4 summarizes some of the hypothetical points at which PGE_2 could play a role in angiogenesis, and its relationship to other factors.

There is no reason to expect that these small molecules should be the only mediators of neovascularization, for angiogenesis is a complex biological process, which can be controlled at many points. Dissolution of vascular continuity, induction of cell migration, triggering of endothelial cell mitosis, induc-

tion of capillary sprouts, extension of vascular continuity, and reestablishment of blood flow must all occur in an orderly fashion. On the other hand, the fact that PGE_2 in amounts equivalent to those seen in inflammation, in synovial fluid, in leukocytic infiltrates, and in tumor nodules can by itself elicit angiogenesis suggests that the array of factors capable of inducing neovascularization may become reduced to a few key substances.

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1. Folkman J. Tumor angiogenesis. In: Becker FF, ed. Cancer. New York, Plenum Press, Vol. 3:p355, 1976.
2. Folkman J, Cotran R. Relation of vascular proliferation to tumor growth. Int Rev Exp Pathol **16**:207–248, 1976.
3. Sidky YA, Auerbach R. Lymphocyte-induced angiogenesis (LIA): A quantitative and sensitive assay of the graft vs. host reaction. J Exp Med **141**:1084–1108, 1975.
4. Auerbach R, Sidky YA. Nature of the stimulus leading to lymphocyte-induced angiogenesis. J Immunol **123**:751–754, 1979.
5. Polverini PJ, Cotran RS, Gimbrone MA, Jr, Unanue ER. Activated macrophages induce vascular proliferation. Nature (London) **269**:804–806, 1977.
6. Auerbach, R. Angiogenesis-inducing factors: A review. In: Pick E, ed. Lymphokines. New York, Academic Press, Vol 4:p69, 1981.
7. Ben Ezra D. Neovasculogenesis. Triggering factors and possible mechanisms. Surv Ophthalmol **24**:167–176, 1978.
8. Ben Ezra D. Neovasculogenic ability of prostaglandins, growth factors and synthetic chemoattractants. Amer J Ophthalmol **86**:455–461, 1978.
9. Cooper CA, Bergamini MVW, Leopold IH. Use of flurbiprofen to inhibit corneal neovascularization. Arch Ophthalmol **98**:1102–1105, 1980.
10. Owen K, Gomolka D, Droller MJ. Production of prostaglandin E_2 by tumor cells *in vitro*. Cancer Res **40**:3167–3171, 1980.
11. Stenson WI, Parker CW. Prostaglandins, macrophages and immunity. J Immunol **125**:1–5, 1980.
12. Gemsa D. Stimulation of prostaglandin E release from macrophages and possible role in the immune response. In: Pick E, ed. Lymphokines. New York, Academic Press, Vol 4:p355, 1981.
13. Morley J. Role of prostaglandins secreted by macrophages in the inflammatory process. In: Pick E, ed. Lymphokines. New York, Academic Press, Vol 4:p377, 1981.
14. Vane JR. Prostaglandins as mediators of inflammation. Advan Prostaglandin Thromboxane Res **2**:791–801, 1976.
15. Auerbach R, Kubai L, Knighton D, Folkman J. A simple procedure for the long-term cultivation of chicken embryos. Dev Biol **41**:391–394, 1974.
16. Rhine WD, Hsieh DS, Langer R. Polymers for sustained macromolecule release: Procedures to fabricate reproducible delivery systems and control release kinetics. J Pharm Sci **69**:265–270, 1980.
17. Dayer JM, Krane SM, Russell GG, Robinson DR. Production of collagenase and prostaglandins by isolated adherent rheumatoid synovial cells. Proc Nat Acad Sci (USA) **13**:945–949, 1976.
18. Krane SM, Dayer JM. A mononuclear cell factor modulates collagenase and PGE_2 production by rheumatoid synovial cells. In: De Weck AL, Krisensen F, Landy M, eds. Biochemical Characterization of Lymphokines. New York, Academic Press, p439, 1980.
19. Sidky YA, Auerbach R. Response of the host vascular system to immunocompetent lymphocytes: Effects of preimmunization of donor or host animals. Proc Soc Exp Biol Med **161**:174–177, 1979.
20. Roehm NR, Sidky YA, Auerbach R. Lyt phenotype analysis of the effector cells responsible for evoking lymphocyte-induced angiogenesis. Cell Immunol **63**:272–278, 1981.
21. Form DM, Sidky YA, Kubai L, Auerbach R. PGE_2-induced angiogenesis. Prostaglandins and Cancer: First International Conference. New York, Alan R. Liss, p685, 1982.

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Preferential Establishment of a Slowly Dissociable Component in Plasma Membrane Compared to Intracellular Prolactin Receptors (41549)

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Abstract. Prolactin dissociates more readily from rat liver than from rabbit mammary prolactin receptors. The rate of dissociation is dependent in the time of association. In rat liver, prolactin dissociates from receptors at the cell periphery (plasma membrane, PM) more rapidly than those of microsomes, a major component of which is the intracellular Golgi membranes. The dissociation curves following 1 hr of association can be resolved into a fast and slow component by logarithmic transformation, with a greater than twofold increase in the fast componment of the dissociation rate constant (k_2) in PM compared to microsomal membranes. With longer association times (10 hr), plasma membranes develop a more slowly dissociable component, with dissociation characteristics (rate constants) similar to those observed in microsomes following 1 hr of association. Another means of visualizing this difference is by a Scatchard plot of prolactin binding to PM, microsomal, and Golgi membranes. The affinity constants in the microsomal and Golgi fractions are identical whereas that for the PM fractions more than twofold lower. The decreased affinity in the PM would allow prolactin to more readily dissociate from its receptor than from receptors with higher affinity. Although the differences between PM and Golgi receptors observed are small, they are confirmed by direct measurements of affinity constants and dissociation rate constants. Therefore, it appears that receptors with lower affinity at the cell periphery are those involved in the initial mechanism of action of prolactin.

The interaction of a hormone with its receptors is the initial event leading to the formation of a hormone receptor complex. Under equilibrium conditions, this reaction has been considered to be freely reversible (1, 2). However, for an increasing list of hormones, including PRL, hGH, TSH, insulin, LH, and β-adrenergic (3–10), it appears that dissociation of the hormone receptor complex is difficult and that a slowly reversible complex is established with an increase in the time of association of the hormone with its receptor.

The present studies were undertaken to examine differences in prolactin receptor dissociation between two target tissues, rat liver and rabbit mammary gland, and to compare the rate of dissociation between various subcellular binding components.

Materials and Methods. *Animals.* Mammary glands of 6-day lactating rabbits and livers from ovariectomized female Sprague–Dawley rats weighing 200 g, treated for 7 days with 5 μg estradiol-17β, were removed. CB-154 (Sandoz, Basel) was injected just prior to sacrifice in both rabbits and rats to increase receptor content (11).

Membrane preparation. Crude microsomes were prepared from rabbit mammary glands and rat liver by differential centrifugation (10, 12). Purified plasma membranes (PM) were prepared by the method of Ray (13) and Golgi membranes by the method of Bergeron *et al.* (14). The purity of the subcellular fractions was assessed by determining 5'-nucleotidase (15) and galactosyl transferase (16) activities, marker enzymes for plasmalemna and Golgi membranes, respectively. Protein was measured by the Lowry method (17) using bovine serum albumin (BSA) as a standard.

Hormones. Ovine prolactin (oPRL, NIH-P-S13, 35 U/mg) was kindly provided by the Hormone Distribution Program of the NIAMDD, NIH.

Iodination of oPRL. ^{125}I-oPRL was prepared utilizing low concentrations of chloramine-T (500 ng) and 5 μg of oPRL and purified on a column of Sephadex G-75 (18). The specific activity ranged from 40 to 60 μCi/μg. The integrity of the labeled hormone

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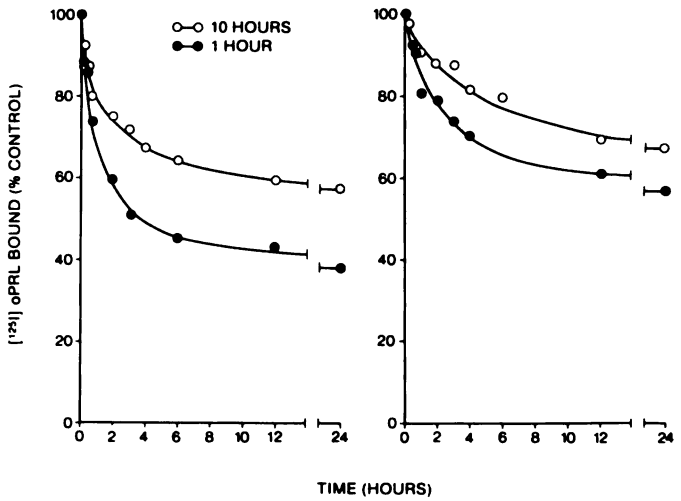

FIG. 1. Dissociation of ^{125}I-oPRL from microsomal membranes of rat liver (left panel) and rabbit mammary gland (right panel) at 23° following either 1 or 10 hr of association of the labeled hormone with the receptor. Specific binding at time 0 for rat liver is 7520, and 18,970 cpm for 1 and 10 hr association, respectively; and for rabbit mammary microsomes, specific binding is, respectively, 17,300 and 38,500 cpm for 1 and 10 hr of association.

at the outset of the studies was verified with a laboratory control receptor preparation of rabbit mammary gland. Labeled prolactin bound >25% of the added hormone with 200 μg of membrane protein and >75% in the presence of excess membranes. Iodinated hormone dissociated from membrane fractions retained its capacity to bind to control receptor preparations.

Assay and dissociation procedures. ^{125}I-oPRL (approximately 100,000 cpm) was incubated with rabbit mammary gland or rat liver membranes (200 μg) for either 14 or 16 hr at 23°, which is the time required for maximal binding to be obtained or for the times indicated in the figures and tables. The final volume was 0.5 ml in an assay buffer of 25 m*M* Tris, containing 10 m*M* $MgCl_2$, and 0.1% BSA. Duplicate tubes, two in the absence and two in the presence of excess (1 μg) oPRL, were used. Bound and free hormones were separated by centrifugation at 2300*g* for 15 min following the addition of 3 ml assay buffer to each tube. Specific binding was the difference between the total counts per minute (cpm) and nonspecific cpm and expressed as cpm or a percentage of the total radioactivity added.

Dissociation (at 23°) of prolactin from its receptor was initiated in tubes in which the labeled prolactin had been allowed to associate with the receptor for 1 or 10 hr by the addition of 3 ml assay buffer containing 10 μg/ml oPRL. The dissociation reaction was stopped by placing the tubes in ice water and immediately centrifuging them at 2300*g* for 15 min at 4°.

Tubes were counted in a LKB gamma spectrometer with a counting efficiency of 65%. For dissociation experiments, results are expressed as a percentage of the radioactivity bound at time 0 (% of control). Affinity constants and binding capacities (N) were calculated by Scatchard analysis (19) under equilibrium conditions.

Results. The difference in the dissociability of prolactin from rat liver and rabbit mammary gland prolactin receptors is shown in Fig. 1. It can be seen that ^{125}I-oPRL dissociates more readily from rat liver (left panel) than from rabbit mammary prolactin receptors as has been previously reported (3, 4, 20). In addition, the longer that a labeled hormone is allowed to associate with its receptor, the more tightly it appears to be bound resulting in the decreased ability to dissociate. This relationship has been established for a number of hormone–receptor systems (3–10).

In rat liver, where dissociability can be more readily measured, there is a marked difference

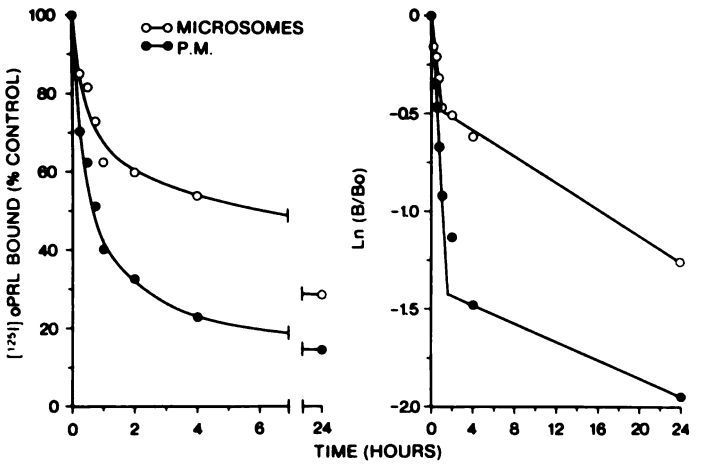

FIG. 2. Dissociation of ^{125}I-oPRL from rat liver microsomes and purified plasma membranes (PM) at 23° following 1 hr of association. The left panel shows the dissociation as a percentage of specific binding at time 0 (after 1 hr of association) which is 11,180 and 8980 cpm for microsomes and PM, respectively. The right panel shows the data transformed to linearize the curve. The slope of these curves is used to calculate the dissociation rate constant (k_2).

a the ability of prolactin to dissociate from eceptors normally found at the cell periphery plasma membrane) than from microsomes, f which a major component in rat liver preparations is the intracellular Golgi membranes 14, 16). Figure 2 shows that ^{125}I-oPRL dissociates from plasma membrane receptors much more readily than from microsomes ollowing 1 hr of association.

The dissociation curves, which can be resolved into fast and slow components based n logarithmic transformation (21), reveal a reater than twofold increase in the faster omponent of the dissociation rate constant k_2) in plasma membranes compared to microsomes with no change in the slow component (Table I).

However, with increased association times, plasma membranes preferentially develop a slowly dissociating component. Figure 3 shows a similar dissociation study carried out with membranes which have been allowed to associate with labeled prolactin for 10 hr. The difference between plasma membranes and microsome component is less apparent. It appears that with longer periods of association (10 hr), prolactin receptors in plasma membranes develop dissociation characteristics similar to those observed in microsomes following just 1 hr of association. This shift is confirmed in Table I.

The differerences in dissociation rate constants can be seen by other means. Figure 4 shows a Scatchard plot of PRL binding in

TABLE I. DISSOCIATION RATE CONSTANTS (k_2) OF ^{125}I-oPRL FROM RAT LIVER MICROSOMES AND PURIFIED PLASMA MEMBRANES AS A FUNCTION OF TIME THE LABELED LIGAND WAS ALLOWED TO ASSOCIATE WITH THE BINDING SITES

	Rate constant, k_2 (hr^{-1})			
	1-hr association		10-hr association	
Cell fraction	Fast component	Slow component	Fast component	Slow component
Microsomes	0.44	0.03	0.34	0.02
Plasma membranes	0.91	0.02	0.38	0.03

Note. Rate constants were calculated from the dissociation data shown in Figs. 2 and 3 (21). These data are the mean of two separate dissociation experiments.

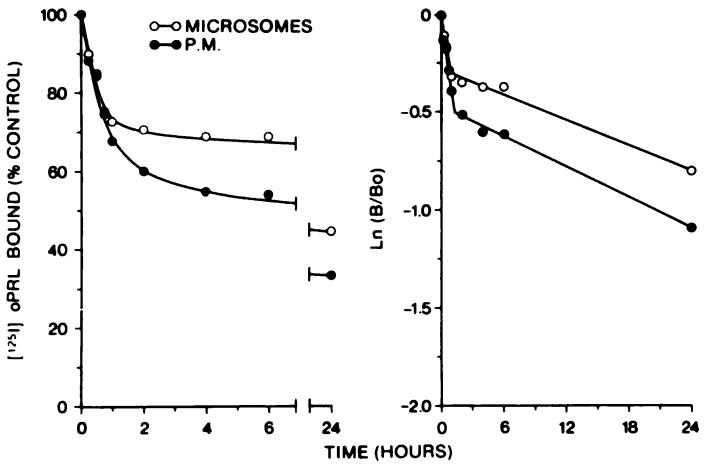

FIG. 3. Dissociation of ^{125}I-oPRL from rat liver microsomes and purified plasma membranes (PM) at 23° following 10 hr of association. Conditions are as described in legend to Fig. 2. Specific binding at time 0 (after 10 hr of association) is 25,800 and 18,380 cpm for microsomes and PM, respectively.

microsome, plasma, and Golgi membranes. As can be seen in Table II, the plasma membrane fraction contains the greatest concentration of 5'-nucleotidase activity and the Golgi intermediate is by far the richest in galactosyl transferase, the affinity constants in microsomes and Golgi fractions are identical, whereas the affinity constant for plasma membranes is slightly more than twofold lower. The decreased affinity in PM would allow prolactin to more readily dissociate from its receptor than from receptors with higher affinities.

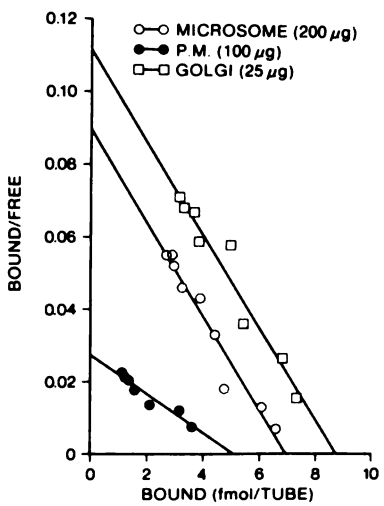

FIG. 4. Scatchard plots of prolactin binding to microsome, plasma membrane (PM), and Golgi membrane fractions.

Discussion. Prolactin more readily dissociates from receptors in rat liver than from receptors in rabbit mammary gland (Fig. 1) as we and others have previously observed (3, 4, 11, 12, 20). As well, there appears to be an increase in affinity with longer association times for mammary receptors as occurs for rat liver receptors. However, since lactating mammary gland preparations are greatly enriched in Golgi membranes (22) and it is difficult to obtain purified plasma membrane fractions from the mammary gland, further comparisons regarding differences between rat liver and rabbit mammary gland have not been made.

These studies could have important implications in terms of cellular function. A very large percentage of prolactin receptors in rat liver is located within the cell (23). The present results confirm the differential subcellular localization of PRL receptors and suggest that receptors in the PM and Golgi are in different forms, with the affinity of the receptor being dependent on the subcellular localization.

Prolactin receptors are down-regulated both *in vivo* and *in vitro* (24, 25) with a passage of the hormone–receptor complex inside the cell (23). The present data suggest that internalization of the hormone–receptor complex is preceded by a tightening of the linkage between the hormone and its receptor, as has

TABLE II. BINDING CONSTANTS OF PROLACTIN RECEPTORS FROM DIFFERENT CELLULAR FRACTIONS OF RAT LIVER CALCULATED BY SCATCHARD ANALYSIS

fraction	5'-Nucleotidase (μmole/hr/mg protein)	Galactosyltransferase (nmole/hr/mg protein)	K_a (nM^{-1})	K_d (nM)	N (fmole/mg protein)
ones	2.6	20.0	6.4	0.16	34.*
ntermediate	14.1	178.0	6.4	0.16	348.4
membrane	34.1	6.3	2.6	0.38	50.8

. 5'-Nucleotidase activity is in units of P_i and galactosyltransferase activity is in units of galactose transferred. lata are the means of two separate experiments.

suggested by Catt (26). The role of in- ization of the prolactin–receptor com- it the present time remains unclear. It ssible it represents simply a scavenger ss for the cell.

llowing the binding of insulin to cell sur- receptors, a ligand-specific internaliza- of the hormone–receptor complex oc- Pastan and his co-workers have char- zed these events in fibroblasts (27) and demonstrated that following internaliza- hormone–receptor complexes form ves- n the cytoplasm which have been iden- as "receptosomes." These structures ap- to accumulate in the Golgi region, and y the hormone–receptor complexes are ded in the lysosomes. Recently, Marshall (28) have reported evidence suggesting following binding of insulin to adipo- there is a degradation in lysosomes, pri- y of the ligand, whereas receptors are ly recycled back to the cell surface. It is sting to speculate that for prolactin re- rs, differences in affinity between recep- it the cell periphery and within the cell t be a reflection of the cellular ability to ate either or both the number of recep- r the degradation of prolactin.

hough the difference in affinity between nd Golgi receptors is small, it is con- d by differences in the rate of dissocia- f labeled hormone from the receptor. It ssible that if a change of affinity occurs ving the binding of prolactin to its PM tors, that it could be related to the pro- on of an intracellular message reponsible e action of prolactin. (29, 30). It appears t is the receptors with the lower affinity l at the periphery of the cell that are most rtant in the initial mechanism of action olactin.

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1. Kahn CR, Freychet P, Roth J, Neville DM. Quantitative aspects of the insulin–receptor interaction in liver plasma membrane. J Biol Chem 249:2249–2257, 1974.
2. De Meyts P, Bianco AR, Roth J. Site interactions among insulin receptors: Characterization of the negative cooperativity. J Biol Chem 251:1877–1888, 1976.
3. Kelly PA, Djiane J, De Léan A. Prolactin–receptor dissociation and down-regulation. In: L'Hermite M, Judd J, eds. Advances in Prolactin, Progress in Reproductive Biology. Basel, Karger, Vol. 6: pp124–136, 1980.
4. Van der Gugten AA, Waters MJ, Murthy GS, Friesen HG. Studies of the irreversible nature of prolactin binding to receptors. Endocrinology 106:402–411, 1980.
5. Donner DB, Martin DW, Sonenberg M. Accumulation of a slowly dissociable peptide hormone binding components by isolated target cells. Proc Nat Acad Sci USA 75:672–676, 1978.
6. Donner DB, Casadei J, Hartstein L, Martin D, Sonenberg M. Characterization of the slowly dissociable human growth hormone binding component of isolated rat hepatocytes. Biochemistry 19: 3293–3300, 1980.
7. Donner DB, Corin RE. Formation of a receptor state from which insulin dissociates slowly in hepatic cells and plasma membranes. J Biol Chem 255:9005–9008, 1980.
8. Powell-Jones CHJ, Thomas CG, Nayfeh SN. Evidence for "compartmentalization" of a slowly dissociable thyrotropin binding component by human thyroid plasma membranes. Endocrinology 104:1136A, 1979.
9. Katikineni M, Davies TF, Huhtaniemi IT, Catt KJ. Luteinizing hormone-receptor interaction in the ter-

tis: Progressive decrease in reversibility of the hormone–receptor complex. Endocrinology **107**:1980–1988, 1980.
10. Ross EM, Maguire ME, Sturgill TW, Biltonen RL, Gilman AG. Relationship between the β-adrenergic receptor and adenylate cyclase: Studies of ligand binding and enzyme activity in purified membranes of 549 lymphoma cells. J Biol Chem **252**:5761–5775, 1977.
11. Djiane J, Durand P, Kelly PA. Evolution of prolactin receptors in rabbit mammary gland throughout pregnancy and lactation. Endocrinology **100**:1348–1356, 1980.
12. Kelly PA, Posner BI, Friesen HG. Effect of hypophysectomy, ovariectomy and cycloheximide on specific binding sites for lactogenic hormones in rat liver. Endocrinology **97**:1408–1415, 1975.
13. Ray TF. A modified method for the isolation of the plasma membrane from rat liver. Biochim Biophys Acta **196**:1–9, 1970.
14. Bergeron JJM, Posner BI, Josefsberg Z, Sikstrom R. Intracellular polypeptide hormones receptors: The demonstration of specific binding sites for insulin and human growth hormone in Golgi fractions isolated from the liver of female rats. J Biol Chem **253**:4058–4066, 1978.
15. Widnell CC, Unkeless JC. Partial purification of a lipoprotein with 5'-nucleotidase activity from membranes of rat liver cells. Proc Nat Acad Sci USA **61**:1050–1057, 1968.
16. Bergeron JJM, Ehrenreich JH, Siekevitz P, Palade GE. Golgi fractions prepared from rat liver homogenates. II Biochemical characterization. J Cell Biol **59**:73–87, 1973.
17. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin–Phenol reagent. J Biol Chem **193**:265–275, 1951.
18. Kelly PA, Leblanc G, Djiane J. Estimation of total prolactin binding sites after in vitro desaturation. Endocrinology **104**:1631–1638, 1979.
19. Scatchard G. The attraction of proteins for small molecules and ions. Ann NY Acad Sci **51**:660–672, 1949.
20. Perry HM, Jacobs LS. Rabbit mammary prolactin receptors: Demonstration of a late puerperal increase in affinity. J Biol Chem **253**:1560–1564, 1978.
21. Williams LT, Lefkowitz RJ. Theory of ligand–receptor interactions. In: Williams LT, Lefkowitz RJ, eds. Receptor Binding Studies in Adrenergic Pharmacology. New York, Raven Press, p27, 1978.
22. Djiane J, Houdebine LM, Kelly PA. Down-regulation of prolactin receptors in rabbit mammary gland: Differential subcellular localization. Proc Soc Exp Biol Med **168**:378–381, 1981.
23. Josefsberg Z, Posner BI, Patel B, Bergeron JJM. The uptake of prolactin into female rat liver: Concentration of intact hormone into Golgi apparatus. J Biol Chem **254**:209–214, 1979.
24. Djiane J, Clauser H, Kelly PA. Rapid down-regulation of prolactin receptors in mammary gland and liver. Biochem Biophys Res Commun **90**:1371–1378, 1979.
25. Djiane J, Delouis C, Kelly PA. Prolactin receptors in organ culture of rabbit mammary gland: Effect of cycloheximide and prolactin. Proc Soc Exp Biol Med **162**:342–345, 1979.
26. Catt KJ, Harwood JP, Aguilera G, Dufau ML. Hormonal regulation of peptide receptors and target cell responses. Nature (London) **280**:109–116, 1979.
27. Pastan IH, Willingham MC. Receptor-mediated endocytosis of hormones in cultured cells. Annu Rev Physiol **43**:239–250, 1981.
28. Marshall S, Olfski JM. Characterization of insulin-induced receptor loss and evidence for internalization of the insulin receptor. Diabetes **30**:746–753, 1981.
29. Teyssot B, Houdebine LM, Djiane J. Prolactin induces release of a factor from membranes capable of stimulating β-casein gene transcription in isolated mammary nuclei. Proc Nat Acad Sci USA **78**:6729–6733, 1981.
30. Teyssot B, Djiane J, Kelly PA, Houdebine LM. Identification of a putative prolactin second messenger activity β-casein gene transcription. Biol Cell **43**:81–88, 1982.

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Neuroendocrine Evidence That Tetrabenazine Is a Dopamine Antagonist (41550)

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Abstract. Tetrabenazine is considered to be a reserpine-like drug because of its ability to block dopamine storage in presynaptic vesicles. We used two methods to determine that tetrabenazine is also a dopamine antagonist. Tetrabenazine displaced the specific [^{3}H]spiperone binding to the dopamine receptors of the anterior pituitary, the corpus striatum, and a transplantable rat pituitary tumor with values for 50% displacement (IC_{50}) of about 15 μM. Under *in vitro* conditions, 0.5 to 10 μM tetrabenazine blocked dopaminergic inhibition of prolactin secretion from rat anterior pituitary glands. One, four, and twenty-four hours after a single tetrabenazine injection (30 mg/kg, ip), the serum prolactin changed from 22 ± 9 ng/ml initially, to 450 ± 52, 254.7 ± 10.4, and 9.3 ± 1.1 ng/ml, respectively. Pituitary glands of the treated rats incubated *in vitro* were refractory to dopaminergic inhibition of prolactin release to an extent that was maximal at one hour but inapparent by 24 hours after injection. *In vivo* and *in vitro*, tetrabenazine induces biological responses characteristic of a dopamine antagonist. These actions are independent of the reserpine-like properties of tetrabenazine. The unusual ability of tetrabenazine both to antagonize dopamine and to block presynaptic dopamine storage may provide a new tool for understanding the physiology of dopaminergic systems.

Tetrabenazine depletes biogenic amines (1–
to produce an animal model of depression
on which antidepressant drug activity can
evaluated (4, 5). Tetrabenazine is also clin-
lly important in the treatment of involu-
tary movement disorders (6, 7). Because
rabenazine blocks vesicular amine storage
, competitively binds to reserpine receptors
10), and induces reserpine-like behavioral
cts (1), tetrabenazine has been thought to
in a manner similar to reserpine (1, 11).
lirect evidence suggests that tetrabenazine
y also have properties typical of a dopa-
ne antagonist (2, 12–14). Little attention,
wever, has been given to the possibility that
pamine antagonism could contribute to the
served effects of the drug.

We found that tetrabenazine was capable
displacing specific [^{3}H]spiperone binding
dopamine receptors in the porcine anterior
uitary (15–18), rat corpus striatum (15), and
at transplantable pituitary tumor (19). In
lition, the ability of tetrabenazine to block
paminergic inhibition of prolactin secre-
n from rat anterior pituitary glands incu-
bated under *in vitro* conditions (20) was demonstrated.

Materials and Methods. *A. Radioligand dopamine receptor binding.* Membranes of rat corpus striatum, porcine anterior pituitary, and the 7315a transplantable rat pituitary tumor, which secretes prolactin and ACTH (21), were prepared for dopamine receptor binding studies as previously described (18). In brief, tissue was quickly dissected after sacrifice and placed into the iced buffer used throughout the study (i.e., 15 mM Tris, 120 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1% ascorbate, 12.5 μM nialamide, 0.1 mM EDTA, pH 7.3). Each tissue was broken with a Polytron (half speed, 15 sec), and the rat striatum and rat tumor were centrifuged at 50,000g for 30 min. The pellet from each tissue was then resuspended in buffer with the Polytron and added to the assay. For the porcine anterior pituitary, after a Teflon–glass homogenization (200 rpm, 15 plunges), an 800g (3 min, three times) centrifugation in 0.32 M sucrose buffer removed nuclei, unbroken cells, and debris. The supernatant was then centrifuged at 110,000g for 60 min and the pellet resuspended for the binding assay in the same way as the striatum and tumor. Because of the

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relatively low density of dopamine receptors in the anterior pituitary (15), this two-step process was adopted to increase the ratio of receptors to protein and thus improve the resolution of the interaction of tetrabenazine with the dopamine receptor.

[^{3}H]Spiperone (1-phenyl-4[^{3}H]spiperone, 51 Ci/mmole, New England Nuclear), a potent dopamine antagonist (22), was utilized to label the dopamine receptors. Nonsaturable binding was defined as that which was present with 2 μM *d*-butaclamol (gift of Ayerst); thus saturable or specific [^{3}H]spiperone binding was that displaced by the excess of *d*-butaclamol. Increasing concentrations of tetrabenazine were added to the displacement experiments run at equilibrium (1.5 hr at 22–23°). The bound [^{3}H]spiperone was separated from free by filtration (Whatman GF/C filter, 15 sec, 20 ml cold buffer) and the trapped radioactivity was assessed by liquid scintillation spectroscopy (machine counting efficiency, 38–43%). The protein content of the assay tubes was determined by a micro method (23), using bovine serum albumin as the standard.

B. The influence of tetrabenazine on prolactin secretion. Female Sprague–Dawley rats (200–220 g, Flow Laboratories, Dublin, Va.) were housed under conditions of controlled illumination, temperature, and humidity with free access to water and standard rat chow.

The synthesis and release of [^{3}H]prolactin ([^{3}H]Prl) were evaluated as previously described (20). Briefly, following decapitation between 08:00 and 10:30 hr, the anterior pituitary glands were rapidly removed and bisected. Three pituitary halves from three different rats were pooled, weighed, and placed into an incubation flask. Each flask contained 1 ml of Medium 199 (M. A. Bioproducts, Walkersville, Md.), and 10 μCi [^{3}H]leucine (40–50 Ci/mmole, Amersham, Arlington Heights, Ill.). Four flasks comprised each experimental group. The flasks were incubated in a Dubnoff shaker under 95% O_2–5% CO_2 at 37° for 5 hr, after which the medium and glands of each flask were separated. The pituitary glands were homogenized in distilled water (1 ml), mixed with a 10% solution of Triton X-100 (100 μl), and after centrifugation, the supernatant was retained. The medium and homogenate from each flask were then separately analyzed by polyacrylamide gel electrophoresis. The radioactivity in the prolactin band was measured in a liquid scintillation spectrometer (Beckman, LS-233) and the incorporated radioactivity was expressed relative to anterior pituitary wet weight as counts per minute [^{3}H]Prl per milligram. The sum of the [^{3}H]Prl values for medium and homogenate represented the total hormone synthesized *de novo* during the incubation. To evaluate the effects on pituitary prolactin synthesis and release, experimental drugs were added directly to the flasks at the onset of the 5-hr incubation.

Dopamine (Sigma) and tetrabenazine methane sulfonate (generously provided by Dr. W. E. Scott of Hoffman-LaRoche, Inc.) were each dissolved in distilled water.

Animals were also given a single intraperitoneal injection of either tetrabenazine 30 mg/kg or the solvent, and trunk blood was collected 1, 4, 16, and 24 hr later. This tetrabenazine dose and temporal pattern of administration were based on profiles established for the observed behavioral and biochemical effects of the drug (1, 2). Sera were frozen until prolactin radioimmunoassay. Anterior pituitary glands of rats given a single injection of tetrabenazine (30 mg/kg, ip) or solvent 1, 4, or 24 hr earlier were then incubated *in vitro* in the absence or presence of 500 nM dopamine, which reliably inhibits prolactin secretion under these conditions (20).

The serum prolactin concentration (ng/ml) was measured by standard double antibody radioimmunoassay, using materials and protocol supplied by Dr. A. Parlow of the NIAMDD Rat Pituitary Hormone Distribution Program, Bethesda, Maryland. All samples from each experiment were analyzed within a single radioimmunoassay and the results were expressed relative to the rat prolactin standard RP-1.

The mean ± SE was calculated for prolactin in the medium and homogenate or serum of each group. Analysis of variance was applied to the data with $P < 0.05$ indicating that a significant difference existed. The dose-dependent nature of dopamine antagonism by tetrabenazine was further evaluated by linear regression analysis.

Results. *A. Tetrabenazine interaction with*

opamine receptor binding. The protein con-
nt in this dopamine receptor assay (i.e., 0.2–
.4 mg protein/tube) was within the range (i.e.,
.1–0.8 mg protein/tube) where specific
H]spiperone binding increased linearly with
ncreasing protein. Specific binding was 60–
0% of total binding and the concentration
f [^{3}H]spiperone used was 0.2–0.4 n*M* (i.e.,
–4× dissociation constant). In each of the
hree tissues, specific [^{3}H]spiperone binding
o the dopamine receptor was displaced by
etrabenazine with values for 50% displace-
nent (IC_{50}) listed in Table I.

*B. The influence of tetrabenazine on pro-
actin release.* We investigated biological
nanifestations of the tetrabenazine interac-
ion with dopamine receptors. As shown in
ig. 1, anterior pituitary glands of normal fe-
nale rats synthesized [^{3}H]Prl equivalent to
5,300 ± 1080 cpm/mg pituitary during a 5-
r incubation. Of this total amount, 9500
± 1150 cpm/mg were secreted into the in-
ubation medium (open bar) and 5800 ± 430
pm/mg were synthesized but retained within

TABLE I. TETRABENAZINE DISPLACEMENT OF SPECIFIC [^{3}H]SPIPERONE BINDING TO THE DOPAMINE RECEPTOR OF THREE DIFFERENT TISSUES

Tissue	IC_{50}[a] (μ*M*)
Corpus striatum	17.4 ± 3.4
Anterior pituitary	14.1 ± 2.3
7315a tumor	16.6 ± 1.7

[a] The IC_{50} was the tetrabenazine concentration that yielded 50% specific [^{3}H]spiperone binding in each experiment. The mean ± SE is given for the three independent experiments performed on each tissue. There were no statistically significant differences between the tissues.

the gland homogenate (shaded bar). Dopamine inhibited [^{3}H]Prl secretion ($P < 0.01$ vs control), led to a small [^{3}H]Prl accumulation within the homogenate and a reduction of total synthesis ($P < 0.05$). Tetrabenazine alone did not influence [^{3}H]Prl synthesis or release, but glands incubated in both dopamine and tetrabenazine together demonstrated that the effects of dopamine were blocked.

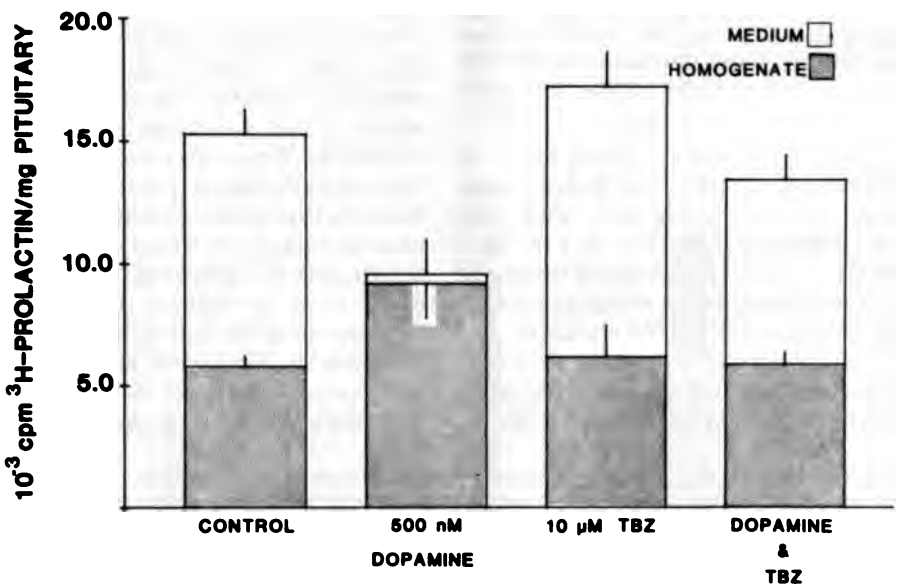

FIG. 1. Tetrabenazine blocks dopaminergic inhibition of [^{3}H]Prl secretion. Three anterior hemipituitary glands from three normal untreated female rats were incubated in a flask in Medium 199 (1 ml) and 10 μCi [^{3}H]leucine for 5 hr. The synthesis and release of [^{3}H]Prl were measured under the influence of dopamine and/or tetrabenazine (TBZ) added to the incubation flasks. The ordinate represents cpm [^{3}H]Prl/mg pituitary as the mean ± SE of the four flasks in each experimental group. The open bars represent [^{3}H]Prl released into the incubation medium and the shaded bars, [^{3}H]Prl synthesized but retained within the homogenate. The sum of the values of medium and homogenate is the total hormone synthesized during the incubation.

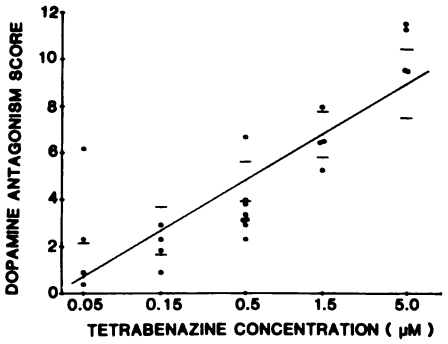

FIG. 2. The dose-related ability of tetrabenazine to reverse dopaminergic inhibition of [^{3}H]Prl secretion. The incubation conditions are described in the Fig. 1 legend. Increasing concentrations of tetrabenazine (shown logarithmically on the abscissa) were added to incubation flasks containing 500 n*M* dopamine. A dopamine antagonism score was derived from the ratio of [^{3}H]Prl released in the presence of tetrabenazine and dopamine compared to the mean value for [^{3}H]Prl released in the presence of dopamine alone, where greater dopamine antagonism by tetrabenazine yielded a greater score. Each point on the graph was the score for an individual incubation flask. The regression line and 95% confidence limits (horizontal bars at each concentration) for the data demonstrated a high correlation of tetrabenazine concentration with dopamine antagonism ($P < 0.0001$; $r^2 = 0.689125$). Dopamine alone inhibited [^{3}H]Prl secretion about 90%.

The effect of 500 n*M* dopamine alone on [^{3}H]Prl secretion was compared with the same dopamine concentration and increasing amounts of tetrabenazine. The data in Fig. 2 demonstrate a highly significant dose-related ability of tetrabenazine to antagonize dopaminergic inhibition of [^{3}H]Prl secretion. Tetrabenazine concentrations greater than 0.15 μM significantly blocked the dopamine effect ($P < 0.05$). Tetrabenazine had no effect on synthesis or release of ^{3}H-growth hormone (data not shown).

The serum prolactin values after injection of either tetrabenazine or solvent are given in Table II. As seen in Fig. 3, one hour after solvent injection, pituitary [^{3}H]Prl secretion could be inhibited by dopamine *in vitro* ($P < 0.01$ compared to control). One hour after injection of tetrabenazine, however, [^{3}H]-prolactin secretion was refractory to dopaminergic inhibition. Prolactin synthesis and release by glands of tetrabenazine injected animals was unchanged from solvent-injected controls. As seen in Fig. 4, tetrabenazine treatment 4 hr earlier blunted the action of dopamine compared to the dopaminergic effect on glands of solvent-injected rats ($P < 0.05$). The anti-dopaminergic effect of tetrabenazine was less prominent at 4 hr than at 1 hr (Fig. 3). Twenty-four hours after a single dose of tetrabenazine, the full inhibitory effect of 500 n*M* dopamine *in vitro* was again apparent. Compared to solvent-injected controls, [^{3}H]Prl secretion was increased in glands of rats given tetrabenazine (30 mg/kg) both 4 and 24 hr earlier ($P < 0.05$).

Discussion. Kuczenski (13) observed two effects after tetrabenazine injection: striatal dopamine synthesis was increased 100% within 15 min, and striatal dopamine content fell 90% by 30 min. In contrast, reserpine never stimulated dopamine synthesis at any dose or time after injection, despite near total dopamine depletion. Increased dopamine synthesis or turnover has been taken as biochemical evidence of pre- and/or postsynaptic dopamine receptor blockade (24, 25). A tetrabenazine analog, Ro 1-9564, also increased striatal dopamine turnover but without causing dopamine depletion, suggesting characteris-

TABLE II. THE SERUM PROLACTIN CONCENTRATION IN FEMALE RATS AFTER TETRABENAZINE ADMINISTRATION

	Interval after injection (hr)[a]			
	1	4	16	24
Control	22 ± 9 (6)	24.5 ± 4.1 (11)	7.9 ± 2.5 (6)	11.9 ± 1.5 (12)
Tetrabenazine	450 ± 52 (12)[b]	254.7 ± 10.4 (10)[b]	30.8 ± 7.1 (5)[b]	9.3 ± 1.1 (11)[c]

[a] Animals were given a single intraperitoneal injection of tetrabenazine (30 mg/kg) or solvent and sacrificed after these four subsequent periods. The values are the serum prolactin concentrations, ng/ml, mean ± SE. The number of animals in the group is in parentheses.

[b] $P < 0.01$ compared with the respective control.

[c] Not significantly different from control.

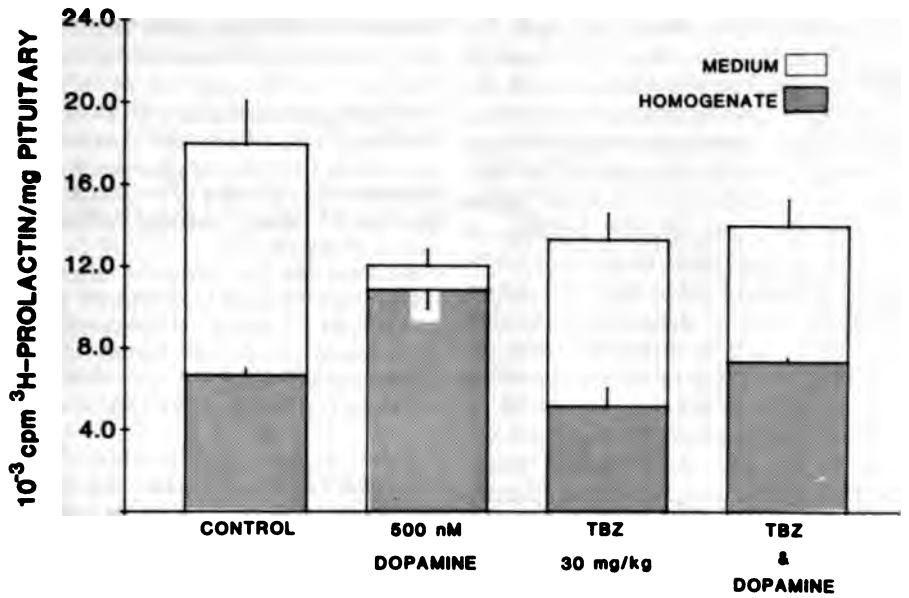

FIG. 3. The influence of tetrabenazine (TBZ) *in vivo* on dopaminergic inhibition of [^{3}H]Prl synthesis and release *in vitro*. Normal female rats were injected with TBZ or solvent and sacrificed one hour later. Their anterior pituitary glands were incubated in [^{3}H]leucine in the presence or absence of dopamine and the values for [^{3}H]Prl synthesis and release were obtained as described in Fig. 1.

of a pure dopamine antagonist (14). Inused dopamine turnover by tetrabenazine also defined clinically by McLellan *et al.* (12), who gave tetrabenazine to patients with Huntington's Chorea and found that the cerebrospinal fluid concentration of homovan-

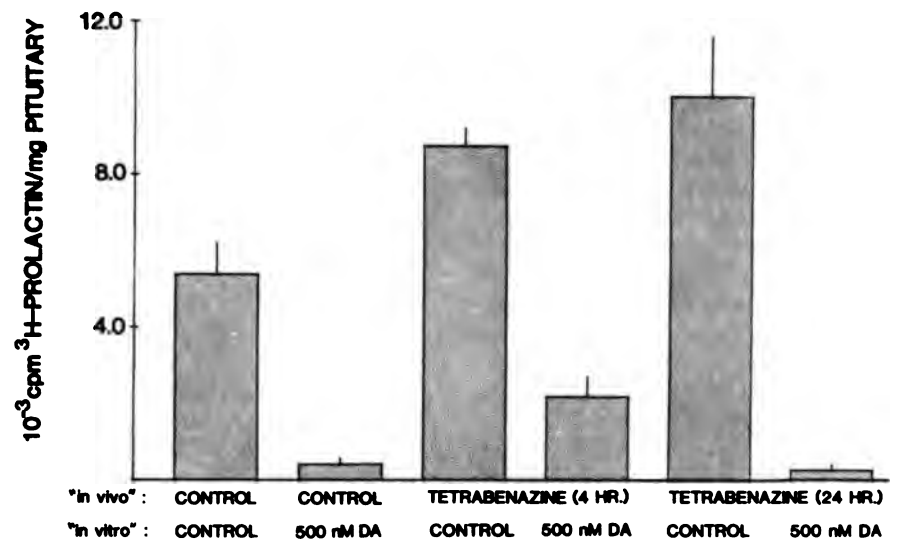
12.0
8.0
4.0
10^{-3}cpm ^{3}H-PROLACTIN/mg PITUITARY
"in vivo" : CONTROL CONTROL TETRABENAZINE (4 HR.) TETRABENAZINE (24 HR.)
"in vitro" : CONTROL 500 nM DA CONTROL 500 nM DA CONTROL 500 nM DA

FIG. 4. The temporal profile of blockade of dopaminergic inhibition of [^{3}H]Prl release *in vitro* by tetrabenazine *in vivo*. Four and twenty-four hours after a single dose of tetrabenazine (30 mg/kg, ip) or solvent, rat anterior pituitary glands were incubated in [^{3}H]leucine in the presence or absence of dopamine. Each bar represents the value for [^{3}H]Prl released into the incubation medium.

illic acid, a dopamine metabolite, was increased. Finally, Pletscher *et al.* (2) reported that tetrabenazine blocked physiological effects of the dopamine agonist, apomorphine.

Our biochemical and neuroendocrine data confirm and extend the impressions that tetrabenazine can antagonize dopamine. Tetrabenazine displaced the specific binding of [^{3}H]spiperone to dopamine receptors in subcellular particulates of three different tissues with an IC_{50} in each of about 15 μM (Table I). Reches *et al.* (25) have reported similar data. Tetrabenazine has a relatively low affinity for the dopamine receptor compared to other dopamine antagonists. We have not yet distinguished whether tetrabenazine competes for the dopamine receptor site directly or modifies receptor function indirectly, such as by altering contiguous membrane properties. Under *in vitro* conditions tetrabenazine blocked dopaminergic inhibition of prolactin release (Fig. 1) in a dose-related manner (Fig. 2) at concentrations that also decreased dopamine receptor binding. One hour after tetrabenazine injection, animals were hyperprolactinemic (Table II), and prolactin secretion was refractory to dopaminergic inhibition *in vitro* (Fig. 3). Thus, *in vivo* dopamine receptor blockade appears to have been at least partially responsible for the hyperprolactinemia. This impression is supported by the observation that 30 min after tetrabenazine (5 mg/kg) prolactin levels were increased 40-fold and prolactin secretion could not be reduced by apomorphine (26). We observed that dopamine antagonism was still apparent 4 hr after one dose of tetrabenazine (Fig. 4) but was of smaller magnitude than at 1 hr. It is important to recognize that metabolites formed after *in vivo* administration of tetrabenazine (1) might also possess anti-dopaminergic properties in the pituitary gland. Tetrabenazine had no direct effect on the basal secretion of [^{3}H]Prl or ^{3}H-growth hormone, suggesting a specificity to its action. Tetrabenazine has also antagonized dopaminergic actions as measured by anterior pituitary secretion of radioimmunoassayable prolactin (27).

Tetrabenazine is thus apparently able to interrupt dopaminergic transmission by two independent mechanisms: antagonism at the dopamine receptor and blockade of dopamine storage in presynaptic granules. This unique situation may offer future insights into the physiology of dopaminergic systems.

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1. Quinn GP, Shore PA, Brodie BB. Biochemical and pharmacological studies of RO1-9569 (tetrabenazine), a non-indole tranquilizing agent with reserpine-like effects. J Pharmacol Exp Ther **127**:103–109, 1959.
2. Pletscher A, Brossi A, Gey KF. Benzoquinolizine derivatives: A new class of monoamine decreasing drugs with psychotropic action. Int Rev Neurobiol **4**:275–306, 1962.
3. Fuenmayor LD, Vogt M. Production of catalepsy and depletion of brain monoamines by a butyrophenone derivative. Brit J Pharmacol **67**:115–122, 1979.
4. Glazer HI, Weiss JM, Pohorecky LA, Miller NE. Monoamines as mediators of avoidance-escape behavior. Psychosom Med **37**:535–543, 1975.
5. Sulser F, Watts J, Brodie BB. On the mechanism of antidepressant action of imipraminelike drugs. Ann NY Acad Sci **96**:279–286, 1962.
6. Jankovic J. Treatment of hyperkinetic movement disorders with tetrabenazine: A double blind crossover study. Ann Neurol **11**:41–47, 1982.
7. Toglia JU, McGlamery M, Sambandham RR. Tetrabenazine in the treatment of Huntington's Chorea and other hyperkinetic movement disorders. J Clin Psychol **39**:81–87, 1978.
8. Ross, SB, Renyi AL. In vitro inhibition of ^{3}H-noradrenaline uptake by reserpine and tetrabenazine in mouse cerebral cortex tissues. Acta Pharmacol Toxicol **24**:73–88, 1966.
9. Manara L, Garattini S. Time course of ^{3}H-reserpine levels in brains of normal and tetrabenazine pretreated rats. Eur J Pharmacol **2**:139–141, 1967.
10. Alpers HS, Shore PA. Specific binding of reserpine. Biochem Pharmacol **18**:1363–1379, 1969.
11. Jankovic, J. Drug-induced and other orofacial-cervical dyskinesias. Ann Intern Med **94**:788–793, 1981.
12. McLellan DL, Chalmers RJ, Johnson RH. A double-blind trial of tetrabenazine, thiopropazate and placebo in patients with chorea. Lancet **1**:104–106, 1974.
13. Kuczenski R. Differential effects of reserpine and tetrabenazine on rat striatal synaptosomal dopamine

biosynthesis and synaptosomal dopamine pools. J Pharmacol Exp Ther 201:357–367, 1977.
14. Saner A, Pletscher A. A benzo (a) quinolizine derivative with a neuroleptic-like action on cerebral monoamine turnover. J Pharmacol Exp Ther 203:556–563, 1977.
15. Creese I, Schneider R, Snyder SH. ^{3}H-Spiroperidol labels dopamine receptors in pituitary and brain. Eur J Pharmacol 46:377–381, 1977.
16. Calabro M, MacLeod RM. Binding of dopamine to bovine anterior pituitary gland membranes. Neuroendocrinology 25:32–46, 1978.
17. Caron MC, Beaulieu M, Raymond V, Gagne B, Drouin J, Lefkowitz RJ, Labrie F. Dopaminergic receptors in the anterior pituitary gland. J Biol Chem 253:2244–2253, 1978.
18. Cronin MJ, Weiner RI. ^{3}H-Spiroperidol (spiperone) binding to a putative dopamine receptor in sheep and steer pituitary and stalk median eminence. Endocrinology 104:307–312, 1979.
19. Cronin MJ, Valdenegro CA, Perkins SN, MacLeod RM. The 7315a pituitary tumor is refractory to dopaminergic inhibition of prolactin release but contains dopamine receptors. Endocrinology 109:2160–2166, 1981.
20. MacLeod RM, Lehmeyer J. Studies on the mechanism of the dopamine-mediated inhibition of prolactin secretion. Endocrinology 94:1077–1085, 1974.
21. MacLeod RM, DeWitt GW, Smith MC. Suppression of pituitary gland hormone content by pituitary tumor hormones. Endocrinology 82:889, 1968.
22. Denef C, Follebouckt J. Differential effects of dopamine antagonists on prolactin secretion from cultured rat pituitary cells. Life Sci 23:431–436, 1978.
23. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–252, 1976.
24. Glowinski J, Scatton B, Julou L. Acute and chronic treatment with neuroleptics: Similarities and differences in their action on nigrostriatal, mesolimbic and mesocortical dopaminergic neurons. Advan Biochem Psychopharmacol 16:617–624, 1977.
25. Reches A, Burke RE, Hassan M, Jackson V, Fahn S. Tetrabenazine may interact with dopamine receptors in rat brain. Trans Amer Soc Neurochem 13:391, 1982.
26. Reches A, Burke RE, Kuhn C, Hassan M, Jackson V, Fahn S. Tetrabenazine blocks dopaminergic receptors in rat brain. Ann Neurol 12:94, 1982.
27. Login IS, Cronin MJ, MacLeod RM. Tetrabenazine has properties of a dopamine receptor antagonist. Ann Neurol 12:257, 1982.

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Role of Nocturnal Feeding in the Development of the Diurnal Rhythm of Jejunal Sucrase Activity (41551)

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Abstract. A diurnal rhythm of jejunal sucrase activity has been shown previously to make its developmental appearance in the rat at the time of weaning (Day 22). In this study we found that the rhythm was not present on Day 23 if the onset of feeding was not coordinated with the onset of darkness. Conversely, the sucrase rhythm appeared precociously (Day 19) in pups weaned onto chow on a schedule in which the onset of feeding is coordinated with the onset of the dark period. It is concluded that the normal developmental appearance of the sucrase rhythm is due, at least in part, to the fact that *ad libitum* feeding becomes nocturnal around Day 22.

In adult rats, numerous digestive and absorptive activities of the jejunum exhibit circadian rhythms. Disaccharidases have been the most widely studied of these activities. In rats fed *ad libitum*, maltase and sucrase activities peak during the dark period of the light/dark cycle (1, 2). If feeding is restricted to the light period, the phase of the enzyme rhythms changes such that maximal activities coincide with the time of feeding (1, 2). Likewise, when adult rats are kept in continuous light or continuous darkness, peak values of maltase and sucrase activities occur at the time of feeding (3, 4), even when food is offered at unusual intervals such as every 12 hr (3) or every 32 hr (5). Thus, in the adult, the rhythms of these disaccharidase activities are synchronized more strongly by the feeding rhythm than by the lighting rhythm. Surprisingly, it is the protein component of the diet rather than the carbohydrate which elicits disaccharidase rhythms (3, 6). Furthermore, these enzyme rhythms cannot be a direct consequence of rhythmic availability of nutrients in the lumen because maltase and sucrase activities continue to show a rhythmic pattern in fasted animals in continuous light (5), in bypassed jejunal sacs (6), and in animals receiving intravenous nutrition (7). Thus it is likely that the rhythmic changes in disaccharidase activities in adult rats are generated by an endogenous circadian time-keeping mechanism and the time of feeding merely sets the phase of the enzyme rhythms.

During development, disaccharidase rhythms are not present in fetal rats (8) or in suckling rats (9, 10). When rat pups are allowed *ad libitum* access to both their dam and solid food, the sucrase rhythm is first detected on the 22nd postnatal day (9), coincident with the time that weaning is normally completed. When animals are prevented from weaning, no rhythm of jejunal sucrase activity is present on Day 22 (9). This observation led to the conclusion that the ontogenic appearance of the sucrase rhythm is cued by some aspect of weaning (9). The overall aim of the current study was to further elucidate this role of weaning.

The process of weaning constitutes a complex environmental change for the intestinal tract. The diet changes in composition and there is a significant change in the rhythmic pattern of *ad libitum* ingestion. Suckling rats normally feed predominantly during the light period (11, 12), despite the fact that they seem to be intrinsically nocturnal feeders (13). Diurnal suckling is probably imposed on the pups by the mother as a result of her nocturnal pattern of feeding and activity. Only when the pups begin to nibble chow can they express their own nocturnal feeding rhythms (9, 11). Thus, one potentially critical aspect of normal weaning is that the onset of feeding becomes coincident with the onset of the dark period. In this regard it is important to note that the study in which weaning was prevented (9) utilized a suckling schedule wherein the onset of feeding was not coordinated with the onset of the dark period. The aim of this work was to clarify the role of the timing of

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eeding rhythm with respect to the devel-
ental appearance of the circadian rhythm
junal sucrase activity.

aterials and Methods. *Chemicals.* Su-
: used for assay of sucrase was ultra pure
e (Schwarz-Mann, Orangeburg, N.Y.).
:ose was measured using Statzyme Glu-
(500 nm) from Worthington Biochemi-
(Freehold, N.J.). It was necessary to dis-
: this reagent in 500 m*M* Tris, pH 7.4
ead of water), in order to inhibit residual
ise activity. Bovine serum albumin (Sigma
nical Co., St. Louis, Mo.) was used as the
:in standard. All other chemicals were re-
t grade.

nimals. Pregnant rats of the Sprague–
ley strain (Charles River CD) were re-
:d from Charles River Breeding Labora-
s (Wilmington, Mass.) on the 15th day
:station. They were housed in air-condi-
:d quarters (21° + 1°) with a 12-hr light/
: cycle. On the due date, cages were
ked every 2 hr for the presence of pups.
birthdate was regarded as Day 0. Ap-
imately 24 hr postpartum, all litters were
:ed to nine pups. Each litter was housed
its mother with food (Purina Lab Chow)
water available *ad libitum* until the age
n experiments were begun.

ood intake. Preliminary studies showed
for a 4-hr feeding period, the weight of
nach contents accurately reflected the
ht of food ingested. In addition, we found
precociously weaned pups were much
e likely to thrive if they were housed in
ps of four or eight than if they were housed
ly. Because measurement of individual
l intake would require single housing, we
:rred to house pups in groups and to use
nach contents as a measure of ingestive
ıvior.

xperiment 1A. Three litters of nine pups
: used for this experiment. At 20 days of
mothers were removed during the first 3
f the light period. From that time onward,
s were provided with solid food (Purina
Chow) during the entire dark period and
he first 4 hr of the light period. Thus, on
schedule the onset of feeding was coor-
ted with the onset of the dark period. Food
removed during the latter 8 hr of the light
od. Water was available at all times. The
ifice sequence began during the light pe-
on the 22nd day of age. One pup was removed from each litter every 4 hr until all nine pups in each litter had been sacrificed. To avoid disturbances in the animal quarters at the time of the light/dark and dark/light transitions, the time points were arranged to occur 0.5 hr after lights-off and lights-on. At each time point, pups were weighed and then sacrificed by decapitation. Stomach contents were removed and weighed, and the small intestine (from the ligament of Treitz to the ileocecal junction) was removed. The proximal half of the small intestine (defined as the jejunum) was flushed thoroughly with ice-cold 0.9% NaCl, then wrapped in Parafilm (American Can Co., Greenwich, Conn.) and stored at −10°.

Experiment 1B. Three litters of nine pups were used for this experiment. Procedures were exactly as described for Experiment 1A except that from Day 20 onward, the food was made available during the last 4 hr of the dark period and through the entire light period; i.e., pups were without food during the first 8 hr of the dark period. Thus on this schedule the onset of feeding occurred 8 hr after lights-off.

Experiments 2A and 2B. The details of these experiments were basically the same as those of Experiment 1A and 1B, respectively, except that mothers were removed on Day 17, the rhythmic availability of chow was instituted at that time, and the sacrifice sequence began on Day 19. Moreover, because weaning at such an early age is known to have deleterious consequences for the pups (14), each litter was given an "aunt" at the time that the mother was removed. "Aunts" were virgin females who were trained on the appropriate feeding schedule for 1 week prior to use in the experiments. Since weanling rats mimic the feeding behavior of adults placed with them (15), the presence of trained "aunts" could be expected to minimize the time of adaptation to the solid diet and to the appropriate feeding schedule.

Assay procedures. Sucrase was assayed in the mid-jejunum where it has maximal activity (16). For this purpose, the middle 10 cm of the jejunum was slit lengthwise and scraped with a steel spatula to remove the mucosa. Homogenates of mucosa were prepared in 9 vol 0.154 *M* KCl by nine strokes (approximately 45 sec) of a Potter Elvehjem Teflon/glass homogenizer. Enzymic activity in 0.1 ml homogenate was measured by incubating at

37° with 0.4 ml 62.5 m*M* sodium maleate buffer, pH 6.0, containing 0.3 *M* sucrose. The reaction was stopped by precipitation of protein with 0.2 ml of 1.8% $Ba(OH)_2 \cdot 8H_2O$, and the mixture was then neutralized with 0.2 ml 2% $ZnSO_4 \cdot 7H_2O$. Glucose produced from sucrose was measured with glucose oxidase reagent. Corrections were made for endogenous glucose in the tissue and in the substrate. This method for assay of sucrase activity is essentially the same as that originally described by Doell and Kretchmer (17). Protein was determined in duplicate by the method of Lowry *et al.* (18). Sucrase activities were first expressed as micromoles of glucose produced per hour per milligram mucosal protein. Values from each litter were then normalized by the method of Stevenson *et al.* (2). This method involved dividing the individual values by the mean value for that litter, thus setting the daily mean for each litter equal to 1.00. Normalization facilitated comparison of the magnitudes of the diurnal fluctuations irrespective of the absolute activity at a given developmental age.

Statistics. All data were assessed by the use of a two-tailed Mann–Whitney *U* test. For jejunal sucrase activity, two hypotheses were tested. Hypothesis I was that there is a rhythm synchronized by the light/dark cycle. To test this hypothesis, the first two time points of the light period were compared with all other points. If these two points were found to be significantly lower than all others, a rhythm synchronized by the light/dark cycle was considered to be present. Hypothesis II was that there is a rhythm synchronized by the feeding cycle. To test this hypothesis, the last point of the feeding period and the first point of the fasting period were compared with all other points. If these two points were significantly lower than all others, a rhythm synchronized by the feeding cycle was considered to be present. For stomach contents the presence of a rhythm was assessed by comparing the time point in the middle of the fasting period with all other time points. The results of these statistical analyses are shown in Table I.

For comparison of individual means for either sucrase activity or stomach weights, Student's two-tailed *t* test for unpaired values was used.

Results. *Experiment 1A.* When pups were raised from Day 20 on a schedule in which the onset of feeding was coordinated with the onset of the dark period, a distinct rhythm of jejunal sucrase activity was present at 22–23 days of age (Fig. 1). Sucrase activities were highest in the 8-hr period spanning the onset of darkness. They declined during the latter part of the dark period and were minimal 4 hr after lights-on. The first two points in the light period were significantly lower than all other points ($P < 0.001$, both hypotheses, Table I) and the ratio of highest to lowest sucrase activity was 3.3. The weight of stomach contents also displayed a distinct rhythm ($P < 0.001$, Table I). However, this rhythm was out of phase with the sucrase rhythm in the sense that the stomach contents were minimal 4 hr before lights-off whereas sucrase activities were maximal at that time.

Sucrase activities in Fig. 1 have been normalized (see Methods) to facilitate comparison with younger animals (Fig. 2). When the data from Experiment 1A were plotted as absolute values (μmole/min/mg protein), a rhythm superimposable on that shown in Fig. 1A was obtained. The mean activity at the lowest point of the curve was 2.87 ± 0.13 and that at the highest point was 9.53 ± 0.46 μmole/hr/mg protein, respectively.

Experiment 1B. When pups were raised from Day 20 on a schedule in which the onset of feeding did not coincide with the onset of darkness, there was no detectable rhythm of jejunal sucrase activity by 23 days of age (Fig. 1). *P* values given in Table I show that there were no significant variations of sucrase synchronized by either the light cycle (Hypothesis I) or the feeding cycle (Hypothesis II). The absolute activities of sucrase in this experiment ranged from 6.30 ± 0.87 to 10.58 ± 1.45 μmole/hr/mg protein, respectively. The lack of diurnal rhythm of sucrase activity could not have been due to lack of rhythmic feeding or inadequacy of food intake since the weights of stomach contents displayed a significant rhythm ($P < 0.001$, Table I) similar to that of Experiment 1A. In both experiments these weights were minimal 4 hr after food was removed from the cages. In Experiment 1B the ratio of highest to lowest mean weight was 8.1 as compared with 5.1 for Experiment 1A. Moreover, the absolute magnitudes of the stomach weights were just as great as in the previous experiment.

Experiment 2A. At 19 days of age, pups

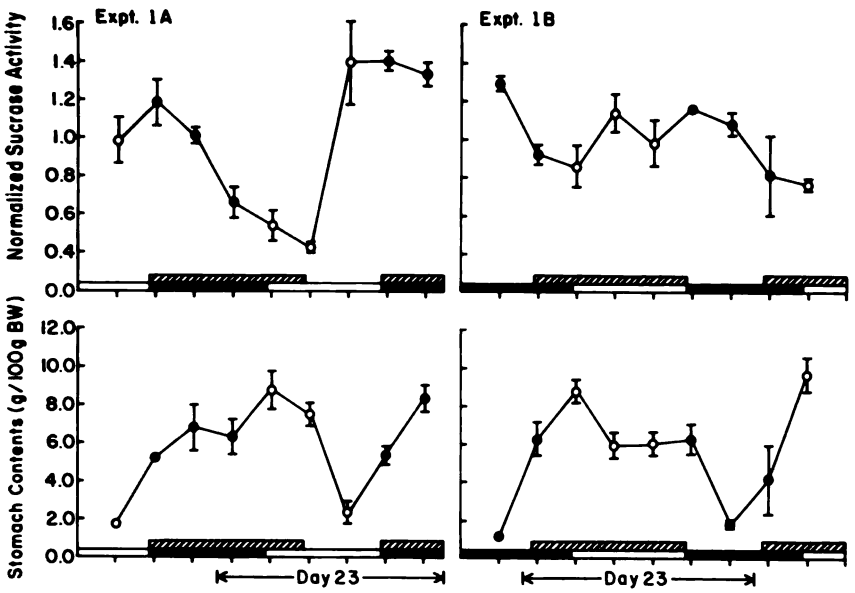

FIG. 1. Jejunal sucrase activity and weight of stomach contents for rat pups aged 22–23 days on either a coordinate feeding schedule (Experiment 1A) or a discoordinate feeding schedule (Experiment 1B). Values are given as mean ± SE (n = 3). Lack of error bars indicates that the SE is smaller than the symbol. Time is shown on the axis by marks every 4 hr and by the age of the pups. Black bars indicate hours of darkness and solid symbols show values for animals sacrificed in the dark whereas open symbols show values for animals sacrificed in the light. Hatched bars indicate the feeding period.

raised with both mother and chow available *ad libitum* do not display a diurnal rhythm of jejunal sucrase activity (9). However, when pups were precociously weaned using a schedule in which the onset of feeding was coordinated with the onset of the dark period (Fig. 2A), a distinct sucrase rhythm was present. The phase of the rhythm was the same as that previously observed with the older pups (Fig. 1A), with minimal sucrase activities occurring during the first 4 hr of the light period and peak activities at the onset of darkness ($P < 0.02$, Table I). However, the ratio of the highest to lowest mean sucrase value in the younger pups (Fig. 2A) was only 1.7, showing that the amplitude of the rhythm was approximately half that found in the older pups (Fig. 1A). This smaller amplitude may be related to the fact that, although there was a feeding rhythm ($P < 0.002$, Table I), the ratio of highest to lowest stomach weights of the younger pups (Fig. 2A) was only 3.5 (as compared with 5.2 for the older pups). In this experiment the absolute values for sucrase activity were 4.60 ± 0.62 μmole/hr/mg protein at the lowest point of the cycle and 7.50 ± 0.68 μmole/hr/mg protein at the highest point of the cycle. The relationship between the phases of the rhythms of stomach contents and jejunal sucrase activity (Fig. 2A) was the same as previously noted for Experiment 1A, i.e., the greatest rise of sucrase activity occurred during the middle 4 hr of the light period while the weights of stomach contents were showing their greatest decline.

Because sucrase values in Figs. 1A and 2A are expressed on the basis of the mucosal protein content, it is possible that the observed sucrase rhythm is due to a converse rhythm

TABLE I. STATISTICAL ANALYSES FOR ALL EXPERIMENTS

Experiments	Sucrase activities		Stomach contents
	Hypothesis I[a]	Hypothesis II[b]	
1A	$P < 0.001$	$P < 0.001$	$P < 0.001$
1B	$P > 0.18$	$P > 0.46$	$P < 0.001$
2A	$P < 0.02$	$P < 0.02$	$P < 0.002$
2B	$P > 0.34$	$P > 0.36$	$P < 0.015$

[a] Hypothesis I is that there is a rhythm synchronized with the light/dark cycle.

[b] Hypothesis II is that there is a rhythm synchronized with the feeding cycle.

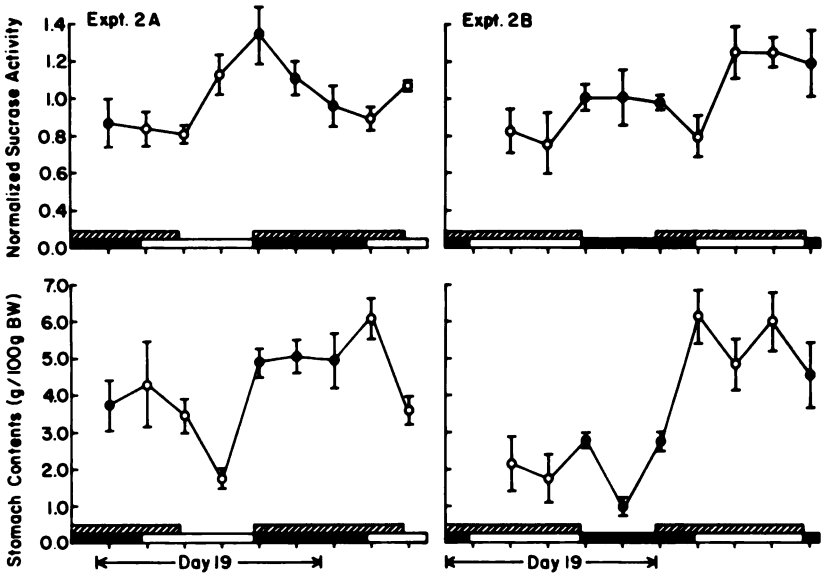

FIG. 2. Jejunal sucrase activity and weight of stomach contents for rat pups aged 19–20 days on either a coordinate feeding schedule (Experiment 2A) or a discoordinate feeding schedule (Experiment 2B). All schematic details of this figure are exactly as described for Fig. 1.

of mucosal protein. However, examination of the protein values (data not shown) indicated that this was not the case. There was, if anything, a tendency toward higher protein values at the time of peak sucrase values, although statistical analysis revealed no significant rhythm ($P > 0.8$) in the mucosal protein content.

Experiment 2B. In accordance with the findings in older pups (Fig. 1B), precociously weaned pups maintained on a schedule in which the onset of feeding was not coordinated with the onset of darkness (Fig. 2B) had no detectable rhythm of jejunal sucrase activity ($P > 0.34$ for both hypotheses, Table I). Absolute values for sucrase activity in this experiment ranged from 4.91 ± 1.07 to 8.09 ± 0.72 μmole/hr/mg protein, respectively. Comparison of the sucrase data with those for stomach contents, indicates that there was no change of sucrase at the time when stomach weights showed their maximal decline. If a sucrase rhythm was cued solely by the feeding rhythm, then the results of Experiments 1A and 2A would predict that a maximal rise of sucrase activity would be temporally coincident with the maximal decline of stomach weights. Although a feeding rhythm did occur in the pups of Experiment 2B ($P < 0.015$, Table I) the absolute weights of stomach contents during the feeding period of Day 19 were significantly lower than those during the feeding period of Day 20 ($P < 0.005$). Moreover, body weights of the pups in Experiment 2B at the time of sacrifice (39.1 ± 0.9 g) were significantly lower ($P < 0.001$) than those of pups in Experiment 2A (46.7 ± 1.1 g). Thus, it is possible that low food intake on Day 19 (and earlier) accounted for the lack of a sucrase rhythm in this experiment. However, it is important to note that even at a later age when the discoordinate feeding schedule was robustly established (Fig. 1B) there was still no sucrase rhythm.

Discussion. The findings from our experiments indicate that the coordination of the onset of feeding with the onset of the dark period is a critical factor in the ontogenic appearance of the diurnal rhythm of jejunal sucrase activity. At 22 days of age a sucrase rhythm is present in rat pups raised in the standard fashion (i.e., allowed *ad libitum* access to both mother and chow until Day 21 and then *ad libitum* access to chow alone) (9). In the current study a more pronounced rhythm was found in 22-day-old pups that were weaned onto chow at Day 20 on a feeding schedule wherein the onset of feeding and darkness were coordinated. However, when these events were not coordinated there was

no sucrase rhythm at 22 days. The efficacy of the coordinated feeding schedule in eliciting rhythmic sucrase activity is illustrated by the fact that the sucrase rhythm appeared early (Day 19) in precociously weaned pups. Thus, during normal development, it seems a key reason for the appearance of the sucrase rhythm between Day 19 and Day 22 is that *ad libitum* feeding of chow becomes nocturnal during this period (9).

In view of the fact that the development of jejunal sucrase activity is powerfully affected by glucocorticoids (17) and that endogenous corticosterone displays a diurnal rhythm beginning around Day 19 (19), it is logical to consider the possibility that corticosterone is responsible for the rhythms reported in this study. We believe this is highly unlikely for three reasons. First, in adult rats the sucrase rhythm is just as robust in adrenalectomized animals as in sham-operated controls (9). Second, the developing intestine has been shown to lose its glucocorticoid responsiveness around days 17–18 (20, 21). Third, Miyabo *et al.* (22) examined the effects of suckling schedule on the development of the corticosterone rhythm in rats and found that the hormone rhythm appeared at 20 days of age, regardless of whether pups were suckled in the light period or in the dark period.

It is apparent that in the adult rat the rhythmic pattern of jejunal sucrase activity is generated by an endogenous circadian oscillator. The strongest evidence for this is the finding that with continuous light and with food offered for 8 hr once every 32 hr the sucrase rhythm has a period of 32 hr, but when these same animals are fasted, the period of the rhythm reverts to approximately 24 hr (5). Assuming that the oscillator is located in the rat brain (23), there must be a neural or hormonal signal which interacts with the intestinal epithelial cells. In the suckling rat the central oscillator seems to be active, as there is an adult-like circadian rhythm of *N*-acetyltransferase activity in the pineal gland as early as the first postnatal week (24–27). Moreover, suckling rats display an intrinsic preference for nocturnal feeding (13). Thus, the lack of disaccharidase rhythms during the suckling period (9, 10) is probably due to lack of the signal mechanism or absence of a coupling between the signal and the oscillator. When feeding becomes coordinated with the natural (nocturnal) phase of the oscillator, the signal mechanism becomes operative. Once the sucrase rhythm is established there is apparently no longer a requirement for coordinated feeding because in adult rats the sucrase rhythm is not abolished when feeding is restricted to the light period (1, 2).

Given the evidence for the significance of coordinate feeding in the ontogenic appearance of the sucrase rhythm, we would have liked to determine whether the nature of the food is important. We were particularly interested in the question of whether nonweaned pups would display a sucrase rhythm if they were forced to suckle on a schedule in which the onset of suckling was coordinated with the onset of the dark period. In the earlier study from this laboratory (9), weaning prevention was achieved by the "16 + 8" suckling schedule originally described by Walker *et al.* (28). On this schedule the dam is removed to a separate feeding cage for the first 8 hr of the dark period and then is returned to the pups for the next 16 hr. The schedule has the advantage of allowing the dam to feed nocturnally as is her preference. In a preliminary experiment in the current series we attempted to modify the "16 + 8" schedule so that each day from Day 11 onward, the dams were removed to a feeding cage during the last 8 hr of the light period. They were then returned to the home cage so that suckling could begin at the onset of the dark period. Starting on Day 19, pups were removed and sacrificed every 4 hr for a 32-hr period. Inspection of stomachs indicated that the pups obtained negligible milk during the first 8 hr of the dark period. This may have been a result of the dams' continuing to be nocturnally active. In this species, milk ejection occurs only when the dam is sleeping (29). In any event, the lack of milk intake by the pups during the early hours of the dark period meant that we were unable to investigate directly the question posed.

In summary, we conclude that the developmental appearance of the diurnal rhythm of jejunal sucrase activity is due primarily to the emergence of a nocturnal feeding pattern in the pups at the time of weaning. Both phasing and periodicity of the sucrase rhythm in the adult rat appear quite labile (5, 6), suggesting a weak coupling between the central pacemaker and the driven oscillators (the sucrase system). From the adaptive standpoint, such a coupling may represent distin

vantage since availability of specific foods at defined times is unlikely in nature. In light of the relatively weak coupling system in the adult rat, it seems even more probable that appearance of a sucrase rhythm during development depends upon congruent input, i.e., phasing of food and light/dark signals.

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1. Saito M, Murakami E, Nishida T, Fujisawa Y, Suda M. Circadian rhythms in digestive enzymes in the small intestine of rats. I Patterns of the rhythms in various regions of the small intestine. J Biochem **78**:475–480, 1975.
2. Stevenson NR, Ferrigni F, Parnicky K, Day S, Fierstein JS. Effect of changes in feeding schedule on the diurnal rhythms and daily activity levels of intestinal brush border enzymes and transport systems. Biochim Biophys Acta **406**:131–145, 1975.
3. Furuya S, Sitren HS, Zeigen S, Offord CE, Stevenson NR. Alterations in the circadian rhythmicity of rat small intestinal functions. J Nutr **109**:1962–1973, 1979.
4. Nishida T, Saito M, Suda M. Parallel between circadian rhythms of intestinal disaccharidases and food intake of rats under constant lighting conditions. Gastroenterology **74**:224–227, 1978.
5. Saito M, Kato H, Suda M. Circadian rhythm of intestinal disaccharidases of rats fed with adiurnal periodicity. Amer J Physiol **238**:G97–101, 1980.
6. Saito M, Sato Y, Suda M. Circadian rhythm and dietary response of disaccharidase activities in isolated rat jejunum. Gastroenterology **75**:828–831, 1978.
7. Stevenson NR, Sitren HS, Furuya S. Circadian rhythmicity in several small intestinal functions is independent of use of the intestine. Amer J Physiol **238**:G203–207, 1980.
8. Stevenson NR, Byra WM, Day SE. Lack of circadian rhythmicity of rat fetal intestinal enzymes as compared to the dams. Dev Biol **60**:487–492, 1977.
9. Beam HE, Henning SJ. Development of the circadian rhythm of jejunal sucrase activity in the weanling rat. Amer J Physiol **235**:E437–442, 1978.
10. Saito M, Suda M, Matzuda H. Postnatal development of circadian rhythms in disaccharidase activities in rat small intestine. Amer J Physiol **234**:E500–503, 1978.
11. Redman RS, Sweney LR. Changes in diet and patterns of feeding activity of developing rats. J Nutr **106**:615–626, 1976.
12. Stern JM, Levin R. Food availability as a determinant of the rats' circadian rhythm in maternal behavior. Dev Psychobiol **9**:137–148, 1976.
13. Henning SJ, Gisel EG. Nocturnal feeding behavior in the neonatal rat. Physiol Behav **25**:603–605, 1980.
14. Lerman R, Koldovsky O. Growth and food intake of prematurely weaned rats: Effect of cortisone and thyroxine injection during the suckling period. J Nutr **109**:916–923, 1979.
15. Galef BG Jr. Social transmission of food preferences: An adaptation for weaning in rats. J Comp Physiol Psychol **91**:1136–1140, 1977.
16. Rubino A, Zimbalatti F, Auricchio S. Intestinal disaccharidase activities in adult and suckling rats. Biochim Biophys Acta **92**:305–311, 1964.
17. Doell RG, Kretchmer N. Intestinal invertase: Precocious development of activity after injection of hydrocortisone. Science **143**:1–2, 1964.
18. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem **193**:265–275, 1951.
19. Ramaley JA. The development of daily changes in serum corticosterone in pre-weanling rats. Steroids **21**:433–442, 1973.
20. Henning SJ, Sims JM. Delineation of the glucocorticoid-sensitive period of intestinal development in the rat. Endocrinology **104**:1158–1163, 1979.
21. Henning SJ, Leeper LL. Coordinate loss of glucocorticoid responsiveness by intestinal enzymes during postnatal development. Amer J Physiol **242**:G89–G94, 1982.
22. Miyabo S, Yanagisawa K-I, Ooya E, Hisada T, Kishida S. Ontogeny of circadian corticosterone rhythm in female rats: Effects of periodic maternal deprivation and food restriction. Endocrinology **106**:636–642, 1980.
23. Stetson MH, Watson-Whitmyre M. Nucleus Suprachiasmaticus: The biological clock in the hamster? Science **191**:197–199, 1976.
24. Ellison N, Weller JL, Klein DC. Development of a circadian rhythm in the activity of pineal serotonin N-acetyltransferase. J Neurochem **19**:1335–1341, 1972.
25. Vanecek J, Illnerova H. The regulation of pineal serotonin N-acetyltransferase activity in newborn rats. Dev Biol **68**:287–291, 1979.
26. Yuwiler A, Klein DC, Buda M, Weller JL. Adrenergic control of pineal N-acetyltransferase activity: Developmental aspects. Amer J Physiol **233**:E141–146, 1977.
27. Brammer M, Binkley S, Enrico J, Duffy M. The rise and fall of pineal N-acetyltransferase *in vitro*: The influence of age. Dev Biol **86**:256–258, 1981.
28. Walker PR, Bonney RJ, Potter VR. Diurnal rhythms of hepatic carbohydrate metabolism during development of the rat. Biochem J **140**:523–529, 1974.
29. Voloschin LM, Tramezzani JH. Milk ejection reflex linked to slow wave sleep in nursing rats. Endocrinology **105**:1202–1207, 1979.

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PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 172, 239–242 (1983)

Critical Period for Neonatal Estrogen Exposure in Occurrence of Mammary Gland Abnormalities in Adult Mice[1] (41552)

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Abstract. There exists a critical period for the development of cervicovaginal lesions in both mice and humans exposed neonatally and antenatally to sex hormones. Mammary glands from year-old female BALB/c mice exposed neonatally to 20 μg estradiol for 5 days commencing at 1 day of age showed the most mammary abnormalities, significantly greater than in controls ($P < 0.005$). The incidence of abnormalities declined when treatment was begun after Day 1. Treatments begun after Day 3 did not result in this structural pattern. Mice ovariectomized after treatment all had inactive mammary glands with no abnormalities. There is a critical exposure period for the later occurrence of mammary gland abnormalities. However, the aberrant secretory state which accompanies these mammary gland alterations may be a consequence of permanent alteration in ovarian function or its endocrine control.

Women may develop clear cell vaginal adenocarcinoma as a result of diethylstilbestrol (DES) exposure *in utero* when the exposure has occurred within the first trimester of pregnancy (1). A critical period also exists for the development of cervicovaginal pathologies in the mouse exposed neonatally to sex hormones. If sex hormone treatment is not administered within the first 3 days after birth, there is a dramatic drop in the incidence of persistent vaginal cornification in mice of the C57BL/Ms strain (2) and of heterotopic columnar epithelium in the upper vagina in mice of the NMRI stock (3). The aim of the present study was to examine the possible existence of a critical period for exposure of the neonatal mouse mammary gland to hormones, analogous to that which exists in the reproductive tract.

Materials and Methods. Five groups, each consisting of about 40 female newborn BALB/cCrgl mice (mammary tumor virus-unexpressed), were given five daily subcutaneous injections of 20 μg 17β-estradiol (E_2) (Cal Biochem, La Jolla, Calif.) in 0.02 ml sesame oil (Hain's cold pressed) or the sesame oil vehicle alone beginning at 1, 2, 3, 5, or 10 days of age. This dose is known to induce ovary-independent changes in the female mouse vagina; owing to binding by α-fetoprotein, large amounts of estradiol must be used to be effective (cf. (4)). About one-half of the mice in each of the 10 treatment groups were ovariectomized at 40 days of age. Mice were maintained at a constant photoperiod of 12 hr light and 12 hr dark and given food (Berkeley Diet—roughly equivalent to other standard rodent chows: at least 20% protein and 7% fat) and water *ad libitum*. All mice were killed at 12 months of age.

Mammary glands attached to the skin were fixed in 10% formalin. After removal from the skin, the glands were stained with iron–hematoxylin and examined blindly as whole-mounts. Subsequently, representative areas of mammary gland were embedded in paraffin, sectioned at 7 μm and stained with hematoxylin and eosin.

Results. Mammary abnormalities were indicative of an aberrant secretory state. The major ducts were often dilated. Dilated portions of both the major and lateral ducts frequently had a beaded appearance and were filled with secretion (Fig. 1), which often appeared white (milk-like) in unfixed glands. The beaded ducts were incompletely segmented structures lined by flattened epithelium surrounding basophilic secretion masses (Fig. 2), histologically resembling dilated lactating

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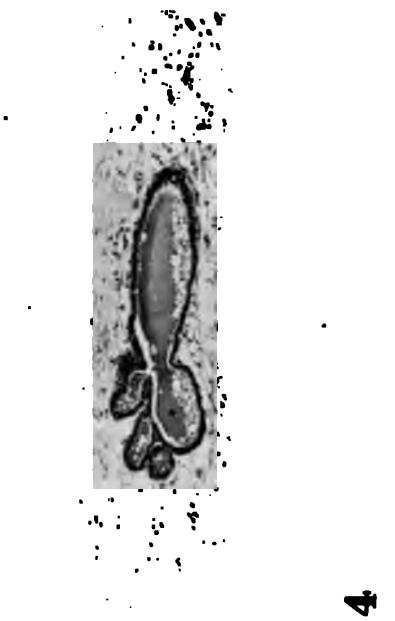

TABLE I. INCIDENCE OF MAMMARY ABNORMALITIES IN 12-MONTH-OLD INTACT FEMALE BALB/cCrgl MICE NEONATALLY TREATED FOR 5 DAYS

Neonatal treatment period[a] (days)	No. of mice with aberrant secretory state (%)		No. of mice with HAN[b]-like lesions (%)	
	Control	Estradiol	Control	Estradiol
1–5	0/22 (0)	6/18 (33)[c]	0/22 (0)	1/18 (6)
2–6	0/23 (0)	1/23 (4)	2/23 (9)	1/23 (4)
3–7	1/23 (4)	2/24 (8)	0/23 (0)	0/24 (0)
5–9	0/21 (0)	0/23 (0)	0/21 (0)	0/23 (0)
10–14	0/20 (0)	0/24 (0)	0/20 (0)	0/24 (0)

[a] Subcutaneous injections of 20 μg 17β-estradiol daily.
[b] Hyperplastic alveolar nodule.
[c] $P < 0.005$ (χ^2).

glands. Although beaded ducts and dilated ducts were often found concomitantly, beaded ducts were also found independently of dilated ducts.

A second abnormality consisted of dilated ductules and endbuds (Fig. 3). Lateral buds were also distended (Fig. 3). The ductules were lined with epithelial cells flattened by the distension of the lumen with secretion (Fig. 4). The extent of secretory abnormality varied within each treatment group from a picture of general stimulation to one of regional stimulation.

Mammary glands from intact mice treated postnatally with estradiol from Days 1–5 had the highest incidence of mammary abnormalities (6/18 or 33%; significantly greater than in controls: $P < 0.005$ by χ^2 test). Treatment periods begun later than Day 1 showed a marked decline in incidence of mammary abnormalities to 8% or less. Only one control female exhibited an aberrant secretory state (Table I).

Hyperplastic alveolar-like dysplasias of unknown significance were also observed in a few animals in both treated and control groups (Table I). All ovariectomized females (15–20 per group), regardless of treatment, had poorly developed, thin ductal systems which frequently occupied only part of the mammary fat pad; no pathological changes were encountered. Accordingly, these negative data were not included in Table I.

Discussion. Treatment of mice with sex hormones prenatally leads to a variety of structural anomalies, such as athelia, amastia, and altered growth rates in early adult life (5–7). Our studies have been concerned with the long-term effects of neonatal estrogen exposure.

Inasmuch as the basic development of the mouse mammary gland occurs during embryonic life, neonatal exposure to exogenous estrogen had little effect on its gross morphological growth pattern. However, neonatal exposure at 1–5 days of age did result in increased incidence of abnormal secretory states (8–10). The incidence of this condition declines with delay in onset of treatment; treatments begun after Day 3 are without visible effect.

Although this experiment was not designed to distinguish between direct and indirect effects of neonatal estrogen on the mammary

FIG. 1. Wholemount of a mammary gland from a 12-month-old BALB/cCrgl intact female given estradiol neonatally on Days 1–5. Segmented or beaded ducts are present. (iron–hematoxylin, 14×)

FIG. 2. Histological section from Fig. 1. Note the segmented ducts and basophilic secretion. (hematoxylin and eosin, 80×)

FIG. 3. Whole mount of a mammary gland from a 12-month-old BALB/cCrgl intact female given estradiol neonatally on Days 1–5. Dilated ductules and end buds are present. (iron–hematoxylin, 14×)

FIG. 4. Histological section from Fig. 3. Note the flattened epithelial cells and distension of the lumen with basophilic secretion. (hematoxylin and eosin, 80×)

gland, the absence of any increase in mammary dysplasias and the atrophic condition of the gland in all ovariectomized females, regardless of treatment, support the hypothesis that the abnormal secretory state is a consequence of long-term alteration in the ovary or its endocrine control. Secondary to abnormal ovarian function, sustained prolactin secretion may occur (4, 11); changes in mammary gland sensitivity as a consequence of the direct effect of the neonatal hormonal stimulation and/or the changed endocrine milieu (4, 12) may also be involved. The critical period may begin in late embryonic life (not examined) but does not last beyond the first 3 days, if indeed beyond the first postnatal day (Table I).

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1. Herbst AL, Bern HA, eds. Developmental Effects of Diethylstilbestrol (DES) in Pregnancy. New York, Thieme-Stratton, 1981.
2. Takasugi N. Cytological basis for permanent vaginal changes in mice treated neonatally with steroid hormones. Int Rev Cytol 44:193–224, 1976.
3. Forsberg J-G, Kalland T. Neonatal estrogen treatment and epithelial abnormalities in the cervicovaginal epithelium of adult mice. Cancer Res 41:721–734, 1981.
4. Bern HA, Talamantes FJ Jr. Neonatal mouse models and their relation to human disease in the female. In: Herbst AL, Bern HA, eds. Developmental Effects of Diethylstilbestrol (DES) in Pregnancy. New York, Thieme-Stratton, Chap 10, p129, 1981.
5. Raynaud A. Foetal development of the mam gland and hormonal effects on its morphogenesi Falconer IR, ed. Lactation. London, Butterw p3, 1971.
6. Hoshino K. Hormonal teratogenesis in mam glands of the mouse. In: Persand TVN, ed. Adv in the Study of Birth Defects, Vol 2, Teratol Testing. Lancaster, MTP Press, p139, 1979.
7. Tomooka Y, Bern HA. Growth of mouse mam glands after neonatal sex hormone treatment. Amer Assoc Cancer Res, Abstract 23, 22:6, 19
8. Jones LA, Bern HA. Longterm effects of neo treatment with progesterone, alone and in co nation with estrogen, on the mammary gland reproductive tract of female BALB/cfC3H Cancer Res 37:67–75, 1977.
9. Jones LA, Bern HA. Cervicovaginal and mam gland abnormalities in BALB/cCrgl mice treated natally with progesterone and estrogen, alone combination. Cancer Res 39:3560–3567, 1979.
10. Mori T, Nagasawa H, Bern HA. Long-term effe perinatal exposure to hormones on normal and plastic mammary growth in rodents: A review. viron Pathol Toxicol 3:191–206, 1979.
11. Kawashima S, Bern HA, Jones LA, Mills KT. tometric study of the pituitary in mice treated natally with steroids and the relationship bet prolactin cells and mammary tumorigenesis. E crinol Japon 25:341–348, 1978.
12. Warner MR, Yau L, Rosen JM. Longterm effe perinatal injection of estrogen and progesteron the morphological and biochemical developme the mammary gland. Endocrinology 106:823–1980.

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Invasion of Burkitt's Lymphoma Cell Lines by *Yersinia enterocolitica* (41553)

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Abstract. We attempted to determine whether or not *Yersinia enterocolitica* could invade two cell lines derived from Burkitt's lymphoma (BL), P3HR-1, and Raji cells, and if the expression of Epstein–Barr virus (EBV) genome could be activated by the invasion of the organisms into the cells. The invasiveness of *Y. enterocolitica* into BL cells was examined morphologically, using transmission and scanning electron microscopy, and the induction of EBV antigens in the BL cells was examined by indirect immunofluorescence. *Y. enterocolitica* was clearly observed to invade P3HR-1 and Raji cells within 2 hr incubation at 37°, after bacterial challenge. However, the invaded BL cells did not show significant induction of EBV early antigen after cultivation for 72 hr at 37°. Although the present experiment failed to yield positive findings pertaining to the activation of EBV genome, our experimental system per se may still be a useful model when attempting to assess the effects of invading bacteria on the viral genome persistently carried in the host cells.

has been reported that a repressed Ep-–Barr virus (EBV) genome in BL cells be activated by halogenated pyrimidines :), sodium butyrate (3), or 12-*O*-tetrade-ɔylphorbol-13-acetate (TPA) (4). The ma-l infections in Africa may be possible co-ɔrs in the induction of Burkitt's lym-ma (5, 6). We consider that other roorganisms may also activate a repressed ' genome in BL cells.

ince the organisms invading BL cells may ɔate a repressed EBV genome, we decided se microbes with the ability to invade hu-ı cells. There are reports on the intracel-r invasiveness of enteropathogenic *Esch-hia coil* (7), *Salmonella* (8), *Shigella* (9), *ɔseudotuberculosis* (10), and *Y. enteroco-a* (11) in human cell cultures. Of these, *nterocolitica* was considered to be a suit-organism as it will invade HeLa cells, ; indicating its pathogenicity (11). We lied the invasiveness of *Y. enterocolitica* BL cells, morphologically using electron roscopy and the effect of the invasion of organisms into BL cells on the expression :BV antigen was examined using an in-ct immunofluorescence method.

Materials and Methods. *Cell lines.* Three lines, P3HR-1, Raji, and HeLa, were used. P3HR-1 cells (12) were producers and were positive for EBV antigens, viral capsid antigen (VCA), and early antigen (EA) by immunofluorescence tests. Raji cells (13) were nonproducers and negative for their EBV antigens. Both P3HR-1 and Raji cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum, penicillin (100 units/ml), and streptomycin (250 μg/ml).

HeLa cells were used as controls. The monolayer cultures were grown in Eagle's MEM medium supplemented with 60 μg/ml of kanamycin and 10% calf serum at 37° for 4 days. After trypsinization, the cells were harvested by centrifugation and resuspended in the same medium, at a concentration of 1×10^6 cells/ml.

Bacterial strain. The *Y. enterocolitica* MYO(X)O-3 strain used in the present study was a gift from Mr. T. Morigaki, Kyoto Prefectural Institute of Hygienic and Environmental Sciences. The organisms were initially grown on an agar plate of brain heart infusion (BHI, Difco) at 27° for 24 hr. One loopful of organisms from the agar plate was then suspended in 10 ml of antibiotic-free Eagle's MEM medium (0.2 mg wet weight of bacteria/ml). The resulting suspension was used for bacterial challenge. In addition, one portion killed by heating at 100° for 10 min served as the control.

Invasion of cell lines by organisms. The

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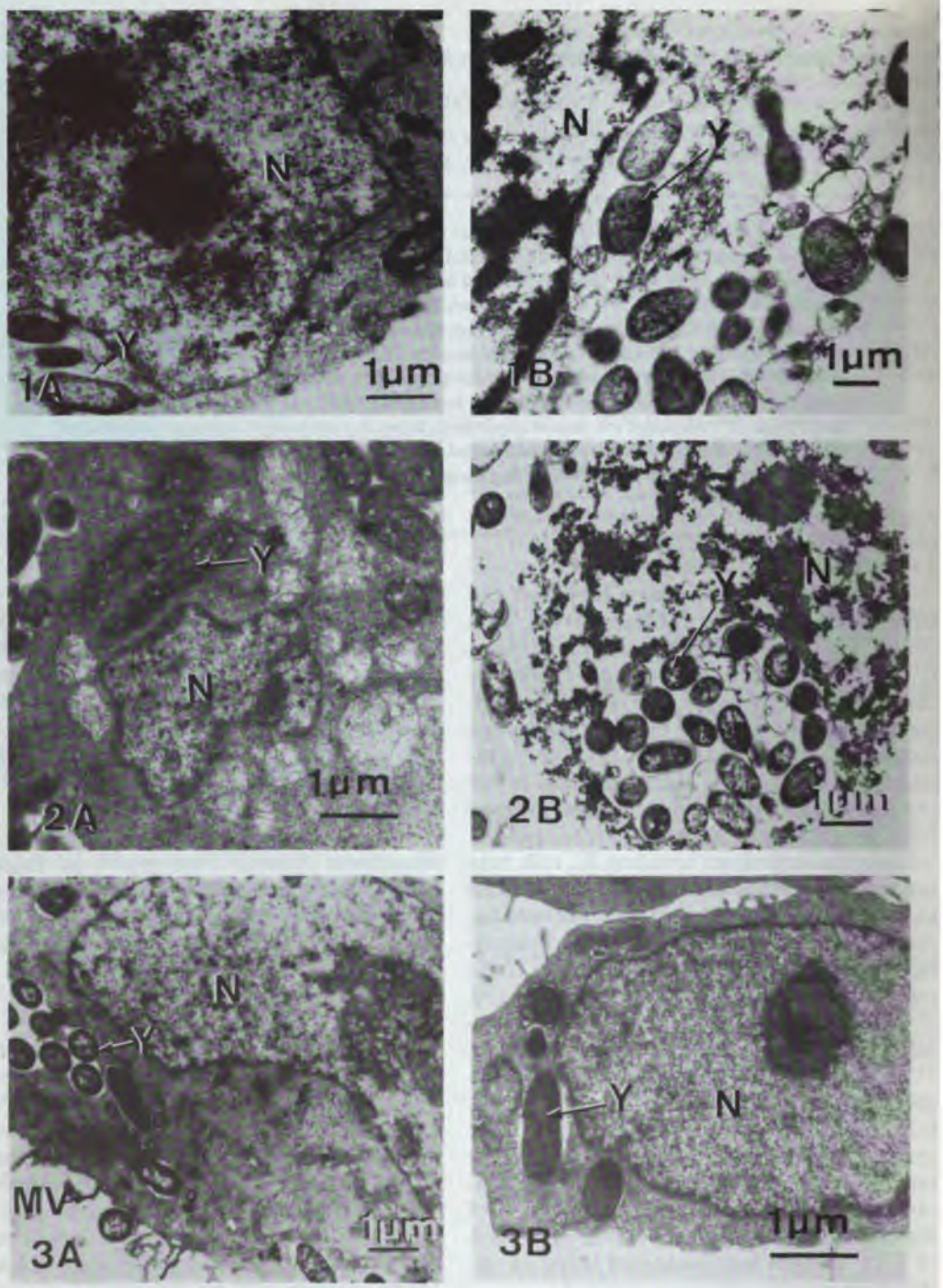

FIG. 1. Transmission electron micrographs showing the presence of *Y. enterocolitica* in the cytoplasm of P3HR-1 cell after bacterial challenge. (A) Incubation period: 2 hr, intracellular *Y. enterocolitica* was not surrounded by a clear peribacillary space. (B) Incubation period: 18 hr, the intracellular *Y. enterocolitica* is present in vacuoles of P3HR-1 and the cell is losing integrity. Y, *Y. enterocolitica*; N, nucleus.

three cell lines, P3HR-1, Raji, and HeLa, were used as target cells. A bacterial challenge experiment was performed using the same procedure as reported by Une (11). Two milliliters of the BL cell suspension containing 1 × 10^6 cells/ml was mixed with an equal volume of the bacterial suspension adjusted to 0.2 mg wet weight of bacteria/ml in antibiotic-free RPMI 1640 medium and the preparation was incubated at 37° for 2 hr. After bacterial challenge, the BL cells were harvested by centrifugation and thoroughly

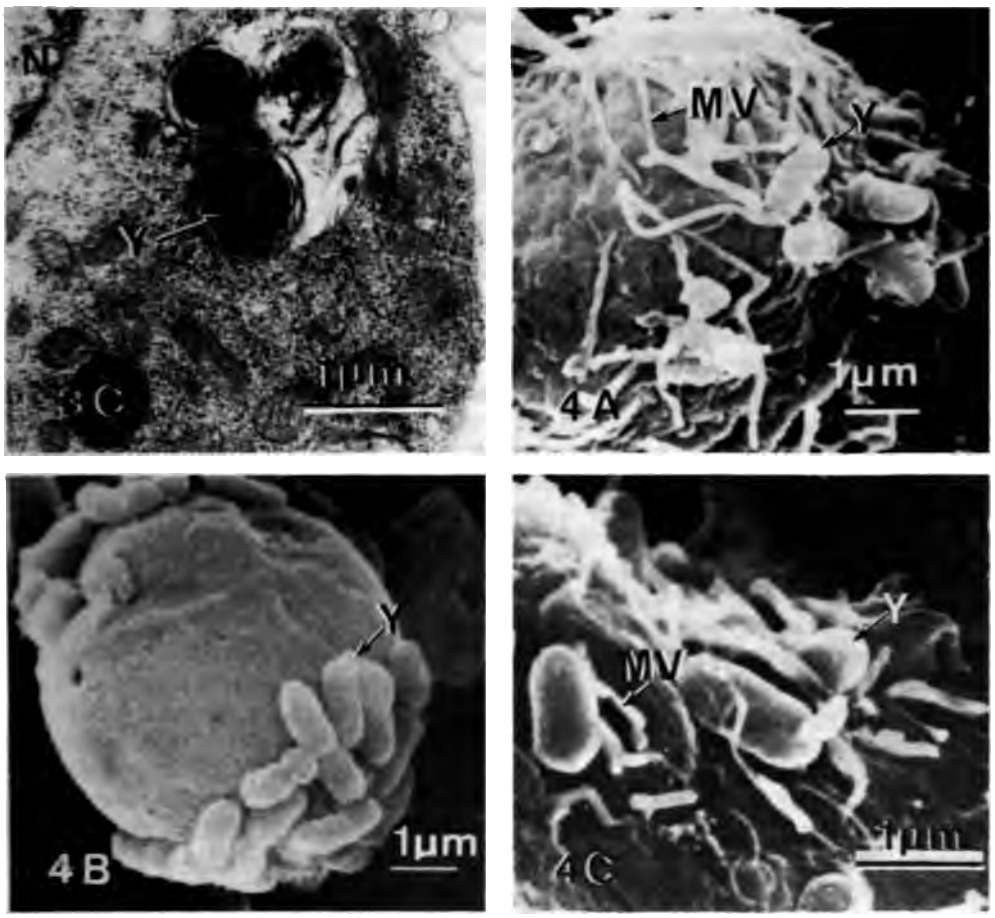

FIG. 2. Transmission electron micrographs showing the presence of *Y. enterocolitica* in the cytoplasm of Raji cell after bacterial challenge. (A) Incubation period: 2 hr, the intracellular *Y. enterocolitica* is not surrounded by a clear peribacillary space. (B) Incubation period: 48 hr, the intracellular *Y. enterocolitica* is present in vacuoles of Raji cell, and the cell is losing integrity. Y, *Y. enterocolitica*; N, nucleus.

FIG. 3. Transmission electron micrographs showing the presence of *Y. enterocolitica* in the cytoplasm of HeLa cell after bacterial challenge. (A) Incubation period: 2 hr, the intracellular *Y. enterocolitica* is enclosed in a phagocytic vesicle. (B) Incubation period: 18 hr, the intracellular *Y. enterocolitica* is digested in part by a phagocytic-like procedure. (C) Incubation period: 72 hr, the disruption of the intracellular *Y. enterocolitica* is proceeding. Y, *Y. enterocolitica*; N, nucleus; MV, microvilli.

FIG. 4. Scanning electron micrographs of *Y. enterocolitica* attached to the three cell lines for 2 hr incubation after bacterial challenge. (A) Attachment of *Y. enterocolitica* to a P3HR-1 cell with microvilli. (B) Attachment of *Y. enterocolitica* to Raji cell without microvilli. (C) Attachment of *Y. enterocolitica* to HeLa cell with microvilli. Y, *Y. enterocolitica*; MV, microvilli.

washed with the same medium to minimize extracellular organisms. The cells were then further incubated for 2, 18, 24, and 48 hr with fresh RPMI 1640 medium containing 5% fetal calf serum and 60 μg/ml of kanamycin to protect the cells from reinfection with the live organisms. The cells were then resuspended in the medium at a concentration of 1×10^6 cells/ml for subsequent electron microscopic observations. With the bacterial challenge experiment using HeLa cells as control, the medium used was Eagle's MEM in place of the RPMI 1640. The challenge procedure and subsequent treatment was carried out in the same order described above, plus an additional 72 hr of incubation.

To determine whether killed *Y. enterocolitica* could invade the three cell lines, the bacterial suspensions were heated at 100° for 10 min and tested for the challenge experiment.

Transmission electron microscopy. The BL cells challenged with the organisms were harvested by centrifugation, fixed by adding 2% glutaraldehyde for 1 hr at 4°, washed twice with 0.1 *M* cacodylate buffer (pH 7.2), and treated with 1% osmiun tetroxide for 1 hr. After postfixation, HeLa cell monolayers were removed from the glass bottle using a rubber policeman and the cells were harvested by centrifugation. The three cell specimens were then dehydrated in a graded series of 30 up to 100% ethanol, cleared in propylene oxide, and embedded in Epon 812. These preparations were sectioned on an LKB 8800 ultrotome III, followed by staining with 10% alcoholic uranyl acetate, 0.1% citrate, and were then examined using JEM 100B transmission electron microscope (JEOL) at 80 kV.

Scanning electron microscopy. The specimens were prepared by the same procedure as described above prior to the dehydration in ethanol. After critical-point drying, they were coated with gold in a JFC-1100 ion sputter (JEOL), and were then examined with a JSM-35C scanning electron microscope (JEOL) at 25 kV.

Immunofluorescence tests. The immunofluorescence was carried out using the method of Henle and Henle (14). P3HR-1 or Raji cells challenged with *Y. enterocolitica* were incubated for 24, 48, and 72 hr at 37°. In the control culture, P3HR-1 cells were adjusted to a concentration of 1×10^6 cells/ml and incubated for the same periods at 37°, with or without 5 m*M* *n*-butyrate (15), and Raji cells were treated with or without 4 m*M* *n*-butyrate plus 25 ng/ml of TPA (16). After incubation, the cells were resuspended in 0.15 *M* phosphate-buffered saline (PBS), pH 7.4, at a concentration of 1×10^6 cells/ml.

A small drop of such suspension was smeared on a slide glass and allowed to dry at room temperature. The smears were then fixed with acetone for 10 min and then dried at room temperature. The smears were incubated for 1 hr at 37° with a 1:40 dilution of standard serum from a patient with nasopharyngeal carcinoma (NPC) (anti-EA titer; 1:1280; anti-VCA, 1:2560). The cell specimens were washed with PBS three times and stained with a 1:20 dilution of FITC conjugated anti-human IgG for 1 hr at 37°. After washing three times with PBS, a cover slip was mounted on the cell smears using 50% glycerol in PBS. Each specimen was examined under a Nikon FT type fluorescence microscope. The induction of EBV antigen-positive cells was examined by counting EA positive in comparison with controls.

Results. *Invasion of cells by Y. enterocolitica.* Observations using the transmission electron microscope revealed that *Y. enterocolitica* invaded the cytoplasm of the P3HR-1 cells within 2 hr after bacterial challenge. Both bacterial and cellular structures were completely maintained for the incubation period (Fig. 1A). On the 18 hr incubation after bacterial challenge, the structures of a few intracellular organisms remained intact, but the cellular structures of P3HR-1 were all but disrupted by that time (Fig. 1B).

In Raji cells challenged with the organisms, the invasion by the organisms occurred within 2 hr of incubation (Fig. 2A). The structures of a few intracellular organisms were stable for 48 hr incubation (Fig. 2B), while the structures of Raji cells began to disrupt. No EBV particles were observed in any of the nuclei or cytoplasms in the P3HR-1 and Raji cells during the first 48 hr of incubation. Slight morphological alteration of the structures of the HeLa cells was observed during the 72-hr incubation after bacterial challenge (Fig. 3C). The electron micrographs showed numerous organisms invading the cytoplasm of the HeLa cells within the first 2-hr incubation (Fig. 3A)

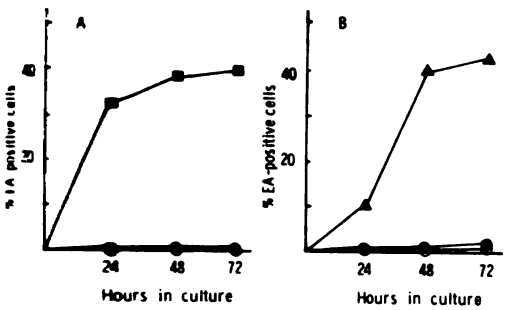

FIG. 5. Effect of invasion of BL cells by *Y. enterocolitica* on the expression of EA. (A) Cultures of Raji cells were incubated (○) without treatment, (●) with *Y. enterocolitica*, and (■) with 25 ng/ml of TPA + 4 m*M* *n*-butyrate. (B) Cultures of P3HR-1 were incubated (○) without treatment, (●) with *Y. enterocolitica*, and (▲) with 5 m*M* *n*-butyrate.

and partial structure disruption of the intracellular organisms by the 18-hr incubation (Fig. 3B). The structures of the organisms were partially disrupted after 72 hr incubation (Fig. 3C).

No organisms were seen in any nuclei of the three cell lines after bacterial challenge. The organisms presented in the BL cells were rarely surrounded by a clear peribacillary space within 2 hr incubation (Figs. 1A and 2A). In contrast, nearly all the organisms observed in the cytoplasms of HeLa cells were surrounded by a clear peribacillary space within the same incubation period. In the case of control studies of the killed organisms, electron microscopy results revealed that the organisms were unable to invade any of the three cell lines during the 48-hr incubation. No morphological alteration of their three cell lines without bacterial challenge was observed during 72 hr incubation.

Scanning electron microscopic findings. The scanning electron microscope was used to visualize the surface features of the three cells during challenging with *Y. enterocolitica*. Organisms were attached to the three cell surfaces without altering the morphological integrity of the cells during the 2-hr incubation after bacterial challenge (Figs. 4A–C). We also observed that the organisms were randomly distributed on the cell surfaces and could be entwined by the microvilli of the P3HR-1 or HeLa cells (Figs. 4A and C). In addition, the micrographs showed that the organisms could attach to the cell surface, even when Raji cell surfaces were smooth and lacked the microvilli (Fig. 4B).

Effect of invasion of BL cells by Y. enterocolitica on the expression of EA. The effect of bacterial challenge on the expression of EA was examined by an indirect immunofluorescence technique. As shown in Fig. 5, the results revealed no significant increases in the percentage of EA-positive cells in both the cultures tested and which were challenged with the organisms.

Figure 5A shows that the EA-positive cells increased little in Raji cultures after bacterial challenge, compared to the untreated control, and the maximum rate of response obtained was 0.1% (72 hr). In the control culture, the positive cells in the Raji cultures treated in combination with 4 m*M* *n*-butyrate and 25 ng/ml of TPA increased 32.2% in the first 24 hr and reached a maximum of 40.1% after 72 hr of continuous treatment. The frequency of EA-positive cells in the untreated cultures was below 0.01%.

Figure 5B shows a slight increase of EA-positive cells in the P3HR-1 cultures after bacterial challenge compared to the untreated control and the maximum rate of response obtained was 1.5% even for up to 72 hr. In the control culture, the treatment of the P3HR-1 cells with 5 m*M* *n*-butyrate markedly increased the number of EA-positive cells and the maximum rate was 43.0% (72 hr), while the frequency of EA-positive cells in the untreated cultures reached a maximum of 0.8% for 72 hr. Attachment of the organisms to the BL cells may slightly increase the number of nonspecific fluorescence-positive cells. Therefore, there is probably little significant difference in the number of EA-positive cells in the BL cells challenged with the organisms, as compared to the untreated controls. A minimum of 500 cells were counted in each preparation.

Discussion. The infectivity of *Y. enterocolitica* to monolayer cell cultures may not be limited to the HeLa cells since its potential invasiveness into porcine kidney cells has been demonstrated by Pedersen *et al.* (17). Thus, the primary purpose of the present experiment was to determine whether or not the organisms were capable of invading the BL cells.

An assessment was made of the fine structure of the BL cells invaded by the organisms and the findings were compared with HeLa cell structures. The transmission electron micrographs showed that the organisms invaded the cytoplasm of the P3HR-1 and the Raji cells within the first 2 hr of incubation, after bacterial challenge. The structures of a few intracellular organisms in the BL cells appeared to be intact during 18 hr incubation after bacterial challenge, while the primary BL cell structures were all but disrupted during the same period. The structures of the HeLa control cells were maintained for 72 hr incubation after bacterial challenge, whereas the structures of the intracellular organisms were partially disrupted during the same incubation.

Heating of the organisms to 100° for 10 min completely inhibited their capability to invade the three cell lines. The HeLa cells are considered to be essentially somewhat phagocytic as it has been reported that there are enclosed small particles such as carbon in the phagocytic vesicles (18, 19), whereas the lymphoblastoid cells such as BL cells are thought to have little phagocytic ability (20). For these reasons, it was considered that the structures of a few intracellular organisms in the BL cells might have been morphologically maintained in a normal state, even when the BL cell structures were disrupted. On the other hand, the structures of the intracellular organisms in HeLa cells seemed to have been disrupted during the phagocytic digestion process. Furthermore, the characteristic feature of the organisms within the BL cells was the predominant absence of an enveloping clear peribacillary space. This may be because the BL cells do indeed have little phagocytic ability. Directly after infection, a few organisms could be detected in the three cell lines and after 18 hr incubation several organisms were present in the cells. These findings suggest that the bacteria multiplied within the cells.

Among the specimens so far examined electron microscopically, no induction of EBV particles was observed in either P3HR-1 or Raji cells, within 48 hr after the bacterial challenge.

In the immunofluorescence tests, the challenge with *Y. enterocolitica* did not significantly increase the number of EA-positive cells either in P3HR-1 or Raji cells, during the 72-hr incubation period.

Although our present experiment failed to yield positive results pertaining to the activation of the EBV genome in BL cells, the experimental system may still be considered to provide a useful model in studying the effects of invasive bacteria on the repressed viral genome.

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1. Sugawara K, Mizuno F, Osato T. Induction of Epstein–Barr virus-related membrane antigens by 5-iododeoxyuridine in non-producer human lymphoblastoid cells. Nature New Biol **246**:70–72, 1973.
2. Gerber P. Activation of Epstein-Barr virus by 5-bromodeoxyuridine in "virus-free" human cells. Proc Nat Acad Sci USA **69**:83–85, 1972.
3. Ragona G, Ernberg I, Klein G. Induction and biological characterization of the Epstein–Barr virus (EBV) carried by the Jijoye lymphoma line. Virology **101**:553–557, 1980.
4. Zur Hausen H, O'Neill FJ, Freese UK. Persisting oncogenic herpes virus induced by the tumour promoter TPA. Nature (London) **272**:373–375, 1978.
5. Salaman MH, Wedderburn N. The immunodepressive effect of a murine plasmodium and its interaction with murine oncogenic viruses. J Gen Microbiol **59**:383–391, 1969.
6. O'Conor GT. Malignant lymphoma in African children. II. A pathological entity. Cancer **14**:270–283, 1961.
7. Dupont HL, Formal SB, Hornick RB, Snyder MJ, Libonati JP, Sheahan DG, LaBrec EH, Kalas JP. Pathogenesis of *Escherichia coli* diarrhea. N Engl J Med **285**:1–9, 1971.
8. Giannella RA, Washington O, Gemski P, Formal SB. Invasion of HeLa cells by *Salmonella typhimurium*: A model for study of invasiveness of *Salmonella*. J Infect Dis **128**:69–75, 1973.
9. Gerber DF, Watkins HMS. Growth of *Shigellae* in monolayer tissue cultures. J Bacteriol **82**:815–822, 1961.
10. Bovallius A, Nilsson G. Ingestion and survival of *Y. pseudotuberculosis* in HeLa cells. Canad J Microbiol **21**:1997–2007, 1975.
11. Une T. Studies on the pathogenicity of *Yersinia enterocolitica*. II. Interaction with cultured cells *in vitro*. Microbiol Immunol **21**(7):365–377, 1977.
12. Hinuma Y, Grace JT. Cloning of immunoglobulin-producing human leukemic and lymphoma cells in long-term cultures. Proc Soc Exp Biol Med **124**:107–111, 1967.
13. Pulvertaft RJV. Cytology of Burkitt's tumour (African lymphoma). Lancet **1**:238–240, 1964.

14. Henle G, Henle W. Immunofluorescence in cells derived from Burkitt's lymphoma. J Bacteriol 91:1248–1256, 1966.
15. Luka J, Kallin B, Klein G. Induction of the Epstein–Barr virus (EBV) cycle in latently infected cells by n-butyrate. Virology 94:228–231, 1979.
16. Lenior G. Unit of biological carcinogenesis: Laboratory studies. In: IARC Annual Report 1979. Lyon. WHO/IARC publication, pp83–84, 1979.
17. Pedersen KB, Winblad S, Bitsch V. Studies on the interaction between different o-serotypes of *Yersinia enterocolitica* and HeLa cells. Acta Pathol Microbiol Scand, Sect B 87:141–145, 1979.
18. Shepard CC. Phagocytosis by HeLa cells and their susceptibility to infection by human *Tubercle bacilli*. Proc Soc Exp Biol Med 90:392–396, 1955.
19. Lee WH, McGrath PP, Carter PH, Eide EL. The ability of some *Yersinia enterocolitica* strains to invade HeLa cells. Canad J Microbiol 23:1714–1722, 1977.
20. Biberfeld P. Endocytosis and lysosome formation in blood lymphocytes transformed by phytohemagglutinin. J Ultrastruct Res 37:41–65, 1971.

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Purification and General Characterization of FAD Synthetase from Rat Liver[1] (41554)

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Abstract. Flavin adenine dinucleotide synthetase (ATP:FMN adenylyltransferase, EC 2.7.7.2) has been enriched more extensively than previously from fresh rat liver. For this, 10% homogenates in sucrose-phosphate buffer were treated with 0.1% Tween-20 prior to high-speed centrifugation to obtain soluble proteins. Those precipitated by 40% saturation with ammonium sulfate were subjected to stepwise addition of calcium phosphate gel to remove pyrophosphatase, and the remaining synthetase was further enriched by passage through a tricalcium phosphate column. An apparent yield of greater than 70% and purification over 70-fold was achieved from the high-speed supernatant fraction. The synthetase activity in solution at 4° was largely lost within a week unless protected by thiols which could partly restore inactivated enzyme. The pH optimum for synthetase activity is near 7.7 when assayed with suitable concentrations of FMN, ATP, and Mg^{2+}. Purified enzyme could be separated into lower (140,000) and higher (325,000) molecular weight components when subjected to molecular sieving on a Sephadex G-200 column.

Earlier literature described the synthesis of flavin adenine dinucleotide from riboflavin *in vivo* in heart, liver, and blood in rats (1) and both *in vivo* and *in vitro* in human blood cells (2). The biosynthesis of the dinucleotide was further studied in rat liver and kidney by using ^{14}C-labeled riboflavin (3, 4). The enzyme responsible for the synthesis of the dinucleotide and pyrophosphate from flavin mononucleotide and ATP was partially purified for the first time from yeast (5). The occurrence of the synthetase (pyrophosphorylase) was also described in higher plants (6). Purification of the dinucleotide-forming enzyme from the rat liver soluble fraction was attempted but with limited success (7), and some of its properties were described (8). The substrate requirements, absolute for adenosine triphosphate and relative for riboflavin 5′-phosphate (FMN), were also reported for crude rat liver FAD synthetase (9). However, the difficulties in isolating the enzyme sufficiently free from FAD-hydrolyzing activity, principally pyrophosphatase, had greatly limited further study and characterization of FAD synthetase, especially from animal tissues.

In the present paper, we describe the means by which significant purification of pyrophosphatase-free FAD synthetase from rat liver can be achieved. Additionally some general characteristics of the enzyme are noted.

Materials and Methods. FMN, ATP, FAD, dithiothreitol, D-amino acid oxidase, cytochrome *c*, ferritin, bovine serum albumin, and polyoxyethylene sorbitan were purchased from Sigma Chemical Company, St. Louis, Missouri. D-Phenyl glycine was obtained from Aldrich Chemical Company, Milwaukee, Wisconsin. Sucrose was the product of Mallincrodt, St. Louis, Missouri. Disodium-EDTA was purchased from Fisher Scientific Company, Pittsburgh, Pennsylvania. Sephadex G-200 and blue dextran were procured from Pharmacia Fine Chemicals, Piscataway, New Jersey. Other chemicals and reagents used were of analytical grade.

Calcium phosphate gel for batch treatment was prepared by the method of Keilin and Hartree (10) and for chromatography by Swingle and Tiselius (11). Protein was determined according to Lowry *et al.* (12) by using bovine serum albumin as the standard. D-Amino acid oxidase apoenzyme was prepared according to the method of Massey and Curti (13). Rat livers were obtained fresh from Sprague–Dawley adult males maintained on laboratory chow.

Assay of FAD synthetase activity. The assay was performed in two steps: (a) Incubation of

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synthetase with FMN and ATP to form FAD and pyrophosphate and (b) quantitation of FAD formed by conversion of apo-D-amino acid oxidase to its active holoenzyme which was subsequently assayed by the spectrophotometric method of Fonda and Anderson (14). For the synthetase reaction, the mixture contained 1.67×10^{-4} *M* FMN, 4.17×10^{-4} *M* ATP, 6.94×10^{-4} *M* magnesium sulfate, and 0.033 *M* potassium phosphate, pH 7.5, to which enzyme was added for 3 ml final volume. The reaction mixture was incubated at 37° and aliquots of 0.3 ml were then withdrawn at 0, 30-, and 60-min intervals and added to 2.59 ml of 0.058 *M* sodium pyrophosphate buffer, pH 8.5. To this was added 10 μl (3–4 μg) of apo-D-amino acid oxidase. After 15 min 5 μmole of D-phenylglycine was added to make a final volume of 3 ml, and the activity of the reconstituted enzyme was monitored at 252 nm for 10–15 min at room temperature against a reagent blank. Under these conditions with excess apooxidase, rate of phenylglycine oxidation was directly proportional to synthetase-produced FAD, as compared to standard amounts of FAD added.

One unit of FAD synthetase is defined as the amount of enzyme that synthesized 1 nmole of FAD at pH 7.5 in one hour at 37°.

Assay of FAD pyrophosphatase activity. The FAD-destroying (pyrophosphatase) activity was determined by incubating at 37° a reaction mixture containing 0.6 μ*M* FAD and 0.5 ml of the enzyme preparation in a final volume of 3 ml of 0.05 *M* potassium phosphate buffer, pH 7.5. From the reaction mixture, 0.3-ml aliquots were withdrawn at the same time intervals as for the FAD synthetase assay, and the pyrophosphatase activity was calculated from the residual FAD as again determined by spectrophotometric assay of the reconstituted D-amino acid oxidase.

One unit of pyrophosphatase is that amount of enzyme responsible for destruction (hydrolysis) of 1 nmole of FAD at pH 7.5 in one hour at 37°.

Synthetase purification. The purification of FAD synthetase includes neutral detergent treatment of a suspension of rat liver homogenate in isotonic medium, centrifugation to obtain the soluble protein fraction, ammonium sulfate fractionation, removal of pyrophosphatase by stepwise addition of calcium phosphate gel, and tricalcium phosphate column chromatography. The protein content and the synthetase and pyrophosphatase activities were determined for every step as detailed in the following: 39 g of rat liver were sliced and homogenized in 0.05 *M* potassium phosphate buffer, pH 7.4, containing 0.25 *M* sucrose, by using the Potter–Elvehjem apparatus with a moderately tight Teflon pestle. The crude homogenate (10%, w/v) showed no FAD synthetase activity due to high masking pyrophosphatase.

The whole homogenate was stirred for 15 min after adding Tween-20 (0.1% final concentration), and was centrifuged at 100,000*g* for one hour in a Beckman Model L preparative ultracentrifuge.

To the clear supernatant, solid ammonium sulfate was stirred to 40% of saturation. The precipitate was recovered by centrifuging at 12,000*g* for 14 min. This was dissolved in 0.05 *M* phosphate buffer, pH 7.4, and dialyzed against three changes of 0.02 *M* buffer over a 15-hr period. The contents remaining in the dialysis bag were centrifuged to remove any precipitate which appeared during dialysis.

Calcium phosphate gel (13.2 mg/ml dry weight) was added to the dialyzed solution at an initial protein:gel ratio of 4. The mixture was slowly stirred for 10 min and then centrifuged (12,000*g*) for 10 min. The slightly yellow supernatant was recovered and the pellet (which contained mostly pyrophosphatase and little synthetase activity) was discarded. The procedure was repeated *four* more times. The clear and faintly yellow supernatant contained virtually all the synthetase and undetectable pyrophosphatase activity.

The supernatant from the preceeding step was poured on a 3.1 × 14-cm tricalcium phosphate gel column preequilibrated with 0.02 *M* phosphate, pH 7.4. The column was washed with 500 ml of the same buffer and then protein eluted with 0.05 *M* phosphate, pH 7.4, in 3.6-ml fractions.

Fully swelled Sephadex G-200 gel capable of sieving proteins of higher molecular weight (above 200,000 daltons) (15) was used. A 2.5 × 50-cm gel column was equilibrated with 0.025 *M* phosphate, pH 7.4. Marker proteins used were cytochrome *c*, bovine serum albumin (dimeric), and ferritin. Blue dextran

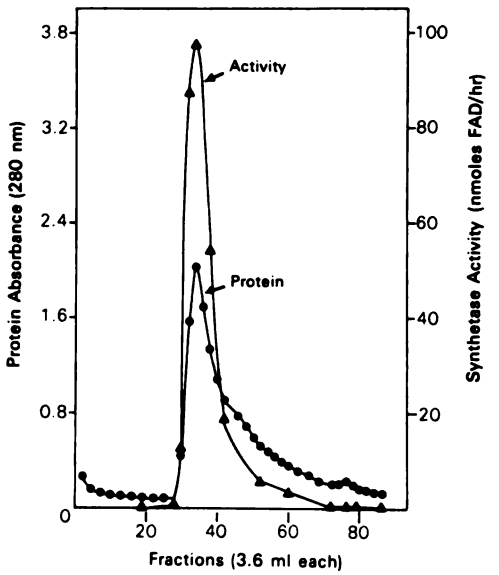


FIG. 1. Elution pattern of FAD synthetase on a tricalcium phosphate column. The fractions (31–36) that contained highest activity were pooled for further analysis. See text for details.

2000 was used to determine the void volume (V_0).

Results. As seen in Fig. 1, synthetase activity coeluted with the maximum of the principal protein fraction initially absorbed on the column. The eluted enzyme was found to be free from pyrophosphatase activity. The absorption spectrum with a principal maximum at 278 nm indicated absence of nucleic acids (A_{260}:A_{280} = 0.58), but a minor chromophoric component is reflected by a small maximum at 438 nm.

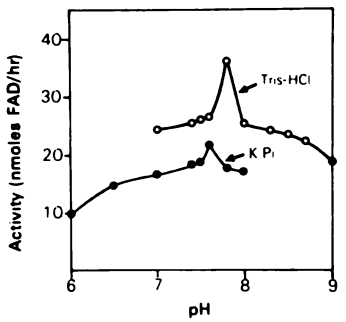
The activities and yields at each stage of purification are summarized in Table I. As seen from the data, much of the activity which was masked by interfering pyrophosphatase at earlier stages is recovered during an apparent enrichment of over 70-fold compared to the original supernatant.

Stability. To assure relative stability, FAD synthetase eluted from the tricalcium phosphate gel column was stored at 0–4° separately and after mixing with 0.2 μmole of FMN, 0.5 μmole of ATP, 1 μmole of magnesium sulfate, and 1 μmole of dithiothreitol in 1-ml portions. All samples unless frozen or treated with dithiothreitol lost most activity within 7 days. A frozen control retained 85%

TABLE I. PURIFICATION OF FAD SYNTHETASE FROM RAT LIVER

Purification stage	Volume (ml)	Protein (mg/ml)	Total protein (mg)	Activity[a] (units/ml)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (*n*-fold)
Tween-treated homogenate	390	21.5	8397	—	—	—	—	—
High-speed supernatant	350	10.8	3780	7.04	2464	0.65	100	1
Dialyzed solution following ammonium sulfate	115	10.1	1162	17.6	2024	1.74	82	2.7
Supernatant from calcium phosphate gel	170	2.5	425	22.9	3893	9.15	158	14.1
Effluent from tricalcium phosphate column	21.6	1.81	39.0	84.9	1835	47.0	74.4	72.4

[a] One unit equals 1 nmole of FAD synthesized per hour in 0.067 *M* potassium phosphate buffer, pH 7.5, at 37°.



. 2. pH dependence of FAD synthetase. Eighteen of enzyme were used in 1.5 ml for the synthetase on at 37°, and 0.3-ml aliquots were taken for assay ial (Methods). Buffers used were either 0.1 *M* po- m phosphate (●) or 0.1 *M* Tris–HCl (○).

e original activity. Dithiothreitol (1 m*M*) nly protected but significantly enhanced times) the synthetase activity examined he second and fourth days. The thiol- ed enzyme even retained 90% of initial ity on the seventh day.

l dependence. The effect of pH and buffer ctivity of FAD synthetase was determined 1 *M* phosphate (pH 6–8) and in 0.1 *M* -HCl (pH 7–9) systems. The pH optima mined with these buffers were 7.6 and respectively (Fig. 2).

olecular weight. Two active components AD synthetase were eluted from each of separate preparations, and their approx- e molecular weights were estimated from ine obtained by plotting the ratio of elu- to void volumes (V_e/V_0) against log mo- ar weights (log MW) of the markers (Fig. 6). The molecular weight of the lighter ponent 1 and the heavier component 2 in the ranges of 130,000–150,000, and 000–339,000, respectively.

scussion. As noted previously (5, 7, 8), synthetase activity could not be readily ted in whole homogenates from rat liver other animal tissues) due to the presence gh amounts of pyrophosphatase. Treat- t of the homogenate with Tween-20 ed to liberate more synthetase and par- retard pyrophosphatase. Greater quan- of this FAD-destroying enzyme were nated in the insoluble cell debris and culates by high-speed centrifugation. ever, pyrophosphatase activity returned when Tween-20 was removed during ammonium sulfate fractionation. The most effective step for removing almost all the pyrophosphatase and much other protein impurities was the calcium phosphate batch treatment which raised the apparent synthetase activity in the supernatant to about 14-fold over that obtained in the soluble fraction (cf. Table I). Tricalcium phosphate chromatography removed more impurities and yielded about 75% of the synthetase with 72-fold purification compared to that measured in the initial soluble protein fraction. Actually, this amount represented about half of the total units of synthetase present in the supernatant following the calcium phosphate batch treatment.

The purified enzyme was faintly yellow in color. When stored at 0–4° it progressively lost most activity within a week. Although freezing offered reasonable protection, dramatic effects were achieved by adding dithiothreitol which not only protected the enzyme from inactivation but also caused reactivation. Preparations that had lost up to half the activity upon storage regained this when treated with millimolar dithiothreitol. This suggests that the thiol can protect sulfhydryl groups of the enzyme that may be essential

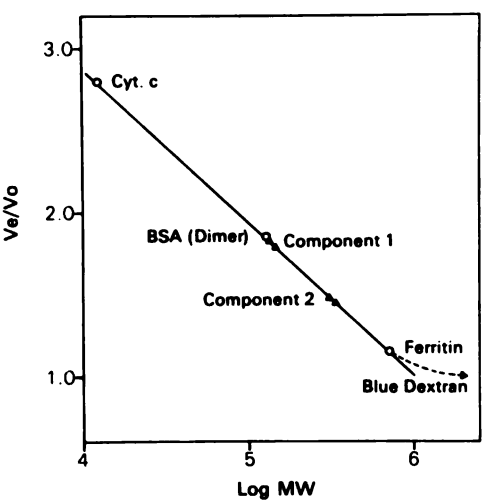


FIG. 3. Molecular weight estimation of FAD synthetase by Sephadex G-200 gel filtration. Two milliliters (1.81 mg/ml for one preparation and 1.73 mg/ml for another) were used for each run. Markers proteins (○) and synthetase preparations (△) are indicated.

for synthetase activity or conformational requirement. Substrates namely FMN, ATP, and/or Mg^{2+}, did not protect the enzyme from inactivation.

The pH dependence curves showed optima at pH 7.6 with phosphate and pH 7.8 with Tris–HCl buffers. Relatively greater activity was observed with Tris–HCl as compared with phosphate which might have complexed some Mg ions required for synthetase activity.

The spectrum of the purified enzyme is typical for a tryptophanyl-containing protein with an absorption maximum at 278 nm. The faintly yellow color attributed to a chromophore with absorption maximum at 438 nm may be due to impurity or possibly a prosthetic group which might be essential for activity. The A_{260}:A_{280} ratio of 0.583 indicated the absence of nucleic acids/nucleotides in the preparation.

Sephadex G-200 gel chromatography allowed separation of two active components of FAD synthetase in roughly equal amounts at the present stage of purification. As the smaller component has an estimated molecular weight of 130,000–150,000 and the heavier component one of 316,00–340,000, it is possible that the synthetase exists in polymeric forms.

1. Ochoa S, Rossiter RJ. CCXLVII. Flavin-adenine-dinucleotide in rat tissues. Biochem J 33:2008–2016, 1939.
2. Klein JR, Kohn HI. The synthesis of flavine-adenine dinucleotide from riboflavin by human blood cells *in vitro* and *in vivo*. J Biol Chem **136**:177–189, 1940.
3. Yagi K, Nagatsu T, Nagatsu-Ishibashi I. Migration of injected ^{14}C-labelled riboflavin into rat tissues. J Biochem (Tokyo) **59**:313–315, 1966.
4. Fazekas AG, Sandor T. Studies on the biosynthesis of flavin nucleotides from 2-^{14}C-riboflavin by rat liver and kidney. Canad J Biochem **51**:772–782, 1973.
5. Schrecker AW, Kornberg A. Reversible enzymatic synthesis of flavin-adenine dinucleotide. J Biol Chem **182**:795–803, 1950.
6. Giri KV, Appaji-Rao N, Cama HR, Kumar SA. Studies on flavinadenine dinucleotide-synthesizing enzyme in plants. Biochem J **75**:381–386, 1960.
7. DeLuca C, Kaplan NO. Flavin adenine dinucleotide synthesis in animal tissues. Biochim Biophys Acta **30**:6–11, 1958.
8. DeLuca C. FAD-forming enzyme (liver). In: Colowick SP, Kaplan NO eds. Methods in Enzymology. New York/London, Academic Press, p342, 1963.
9. McCormick DB. Specificity of flavin adenine dinucleotide pyrophosphorylase for flavin phosphates and nucleotide triphosphates. Biochem Biophys Res Commun **14**:493–497, 1964.
10. Keilin D, Hartree EF. On the mechanism of the decomposition of hydrogen peroxide by catalase. Proc Roy Soc (London) **B124**:397–405, 1938.
11. Swingle SM, Tiselius A. Tricalcium phosphate as an adsorbent in the chromatography of proteins. Biochem J **48**:171–174, 1951.
12. Lowry OH, Rosebrough NJ, Farr AL, Randal RJ. Protein measurement with the Folin phenol reagent. J Biol Chem **193**:265–275, 1951.
13. Massey V, Curti B. A new method of preparation of D-amino acid oxidase apoprotein and a conformational change after its combination with flavin adenine dinucleotide. J Biol Chem **241**:3417–3423, 1966.
14. Fonda ML, Anderson BM. D-Amino acid oxidase. I. Spectrophotometric studies. J Biol Chem **242**:3957–3962, 1967.
15. Andrews P. Gel-filtration behaviour of proteins related to their molecular weights over a wide range. Biochem J **96**:595–606, 1965.
16. Whitaker JR. Determination of molecular weights of proteins by gel filtration on Sephadex. Anal Chem **35**:1950–1953, 1963.

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D-Galactosamine Liver Injury: Absorption of Endotoxin and Protective Effect of Small Bowel and Colon Resection in Rabbits (41555)

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Abstract. D-Galactosamine is an amino sugar with unique hepatotoxic properties in animals. Although the mechanism of liver injury by galactosamine remains controversial, a role for bacterial endotoxin has been suggested. In the present study, using New Zealand rabbits, we show that the significant increase in serum glutamic oxaloacetic transaminase which followed the injection of 4.25 mmole/kg of D-galactosamine was completely prevented in animals subjected to resection of small bowel and colon. Using an immunoradiometric assay specific for *E. coli* 026 endotoxin we showed that after instillation of 50 mg of *E. coli* into the colon, serum levels of this endotoxin were higher in the animals injected with galactosamine than the controls injected with saline. However, the differences in endotoxin concentration between the two groups of animals was statistically significant only at 30 and 60 min. The role of endotoxin in the pathogenesis of galactosamine liver injury is reviewed and discussed.

Since the discovery by Decker and Keppler of the hepatotoxic effects of D-galactosamine hydrochloride in rats (1, 2), this substance has provided a model for the study of fulminant liver necrosis in experimental animals. Galactosamine interferes with protein synthesis in the hepatocytes, but whether this is the mechanism by which the characteristic hepatocellular necrosis occurs has not been clarified. Endogenous gut-derived endotoxin has been suggested (3, 4) as the common mediator of hepatocellular injury induced by a variety of hepatotoxins. In both acute carbon tetrachloride (CCl_4) liver injury (5, 6) and cirrhosis of chronic choline deficiency (7), endogenous lipopolysaccharide (LPS) was shown to be critical for the development of the liver lesion. More recently endotoxin has also been implicated in the pathogenesis of the liver injury induced by galactosamine; colectomy in rats has been reported to protect against liver injury induced by this agent (8, 9). Other studies, however, have challenged the role of gut-derived endotoxin in the production of hepatic injury (10, 11). In this communication we report a similar experimental model to test the role of the intact gut in the development of galactosamine hepatitis, and to detect and quantify the absorption of endotoxin into the systemic circulation after galactosamine administration.

Materials and Methods. *Animals.* New Zealand male rabbits, weighing approximately 3 to 4 lb were used in all of the experiments.

Galactosamine. D-Galactosamine hydrochloride (Sigma Chemical Co., St. Louis, Mo.), was dissolved in phosphate-buffered saline (PBS) with pH adjusted to 6.8 with NaOH. In all experiments the galactosamine was injected into an ear vein at a dose of 4.25 mmole/kg. Control rabbits received a similar volume of PBS.

Endotoxin. Escherichia coli 026 LPS (lyophylized) was obtained from Difco (Detroit, Mich.) and dissolved in isotonic saline.

Immunoradiometric assay for E. coli 026 endotoxin. The assay has been described in detail elsewhere (12). In brief, IgG fraction of rabbit antibody to *E. coli* 026 endotoxin was labeled with ^{125}I. Polyvinyl chloride microtiter V-shaped plates were coated with 50 μl of unlabeled antibody (100 ng/μl) in 0.1 *M* carbonate buffer (pH 9.6) and incubated overnight at 4°. The wells were then washed three times (PBS–Tween 20) and 50 μl of radiolabeled antibody diluted with 1% bovine serum

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albumin (100,000 cpm/50 μl) was added, and the plates were incubated overnight at 4°. Each well was washed five times (PBS–Tween) and counted for 1 min in a γ scintillation spectrometer. A standard curve was plotted on double logarithmic graph paper. This curve plots known concentrations of 026 LPS against the ratio of $E_B/1 - E_B$. (E_B is the endotoxin bound and is derived from the test counts per minute (cpm) minus the blank cpm, divided by the total cpm added). This assay can detect 1 ng/ml *E. coli* 026 endotoxin in serum and shows no cross-reactivity with LPS from other Gram negative organisms.

Enzyme determination. Serum glutamic oxaloacetic transaminase (SGOT) was measured by a modification of the International Federation of Clinical Chemistry method using the DuPont ACA Clinical Analyzer.

Statistical analysis. Enzyme values and *E. coli* 026 endotoxin were plotted against time and were analyzed by Student's *t* test with a confidence level of 95% considered significant ($p < 0.05$).

Experimental design. (1) Evisceration studies. After an overnight fast, rabbits were sequentially numbered and anesthesized in pairs with intravenous sodium pentobarbital. The carotid artery was exposed, and an indwelling cannula inserted for blood pressure monitoring and blood sampling. Four milliliters of blood were withdrawn for determination of baseline SGOT values. The animals assigned an even number had a resection of the small bowel and colon from the third portion of the duodenum to the peritoneal reflection of the sigmoid colon, through a midline incision of the abdomen (evisceration). Due to a common blood supply, the colon could not be resected without producing ischemia in the remaining small bowel. The hepatic artery and portal vein drainage from the spleen, stomach, and pancreas were left intact. The odd-numbered animals had manipulation of the gut without evisceration. The animals were then placed in restraining cages until recovery from the anesthesia. Ten eviscerated and eleven sham-operated animals were injected via an ear vein with 4.25 mmole/kg of D-galactosamine hydrochloride. After galactosamine administration, 4 ml of blood was withdrawn from the carotid artery every 30 min for 3.5 hr or until the animal expired. The mean blood pressure was continuously monitored, and serum samples were analyzed for SGOT.

After termination of the experiment, animals were sacrificed with an overdose of pentobarbital, and wedge liver biopsies were obtained. The liver biopsies were fixed in 10% buffered formaldehyde and stained with hematoxylin and eosin for histological examination.

(2) Absorption studies. Fasted rabbits were anesthesized in pairs and prepared as just described. After obtaining baseline values for SGOT and *E. coli* 026 LPS, a midline abdominal incision was performed and the colon exposed. Fifty milligrams of *E. coli* 026 LPS dissolved in 5 ml of isotonic saline was then injected into the lumen of the colon. Care was taken to avoid extravasation of the solution. The abdomen was closed, and the animals were allowed to recover from anesthesia. The 10 even-numbered animals received 4.25 mmole/kg of D-galactosamine via an ear lobe vein (Group 1). The 10 odd-numbered animals received the same volume of saline intravenously (Group 2). Blood was sampled every 30 min for 210 min for determination of *E. coli* 026 LPS concentration and SGOT activity. At the end of this period the animals were sacrificed with an overdose of sodium pentobarbital, and a wedge liver biopsy was obtained for histologic examination.

Results. *(1) Evisceration studies.* In the sham-operated rabbits given galactosamine (Group 1), there was an elevation of SGOT activity in the arterial blood which began at 90 min and continued throughout the period of observation (210 min). Animals subjected to evisceration showed no elevation of SGOT activity. The difference in SGOT activity between sham-operated and eviscerated animals was statistically significant ($p < 0.05$) at each time period beyond 120 min (Fig. 1).

The mean blood pressure was not different between sham-operated animals and eviscerated animals during the period of observation. Histological examination of the liver biopsies of these animals showed minimal focal necrosis without a consistent pattern in animals of both groups.

(2) Absorption studies. E. coli 026 endotoxin was readily absorbed from the colon into

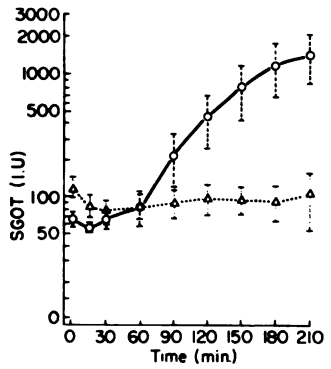

FIG. 1. Serum glutamic oxaloacetic transaminase (SGOT) activity (mean ± SEM) after administration of 4.25 mmole/kg of galactosamine into sham-operated animals (O) and animals with colon and small bowel resection (Δ). Differences between means are statistically significant, $p < 0.05$, at 120 min and thereafter.

the blood of animals given either D-galactosamine or isotonic saline. The difference in concentration of LPS between the two groups of animals was statistically significant only at 30 and 60 min ($p < 0.05$). After 90 min, the concentration of serum endotoxin in both groups was similar (Fig. 2). The SGOT activities were significantly higher than controls ($p < 0.05$) beyond 90 min in the group of rabbits receiving galactosamine (Fig. 2). There was no statistically significant difference in the blood pressures between the two groups of animals.

Discussion. In this study we demonstrate that colectomy and small bowel resection protects the liver against the early toxic effects of galactosamine, which otherwise produces a steady rise in SGOT activity in intact animals within the first 210 min of administration. These studies confirm the work of Liehr and associates who implicated endotoxin in the pathogenesis of this lesion (8, 9, 15, 16). Using the Limulus gelation test, they found that endotoxemia regularly follows the administration of galactosamine in normal rats and that the "endotoxin tolerant" state protected these animals from liver damage (9). In further studies by this group rats subjected to colectomy (9) or partial hepatectomy (17) were similarly protected. Complement-deficient rats (NMRI strain) were also refractory to the toxic effects of galactosamine (8). Based on these findings, they postulated that galactosamine renders the liver incapable of effectively detoxifying endotoxin. In addition, galactosamine was found to cause mast cell degranulation in the gut with local release of histamine, leading to an increase in the colonic absorption of endotoxin. The increased absorption of gut-derived endotoxin will then exert toxic effects on liver cells either directly or through the release of mediators by the Kupffer cells (3, 4, 18–20).

These conclusions, however, have been contested by recent studies. In isolated perfused rat livers where the colon is not a factor, galactosamine exerted a direct toxic effect on hepatocytes (10). Al-Tuwaijri *et al.*, using the Limulus gelation test, failed to confirm the presence of endotoxin in plasma after galactosamine administration in rats (11).

Decker and Keppler (1, 2) extensively studied galactosamine liver injury and have observed an intracellular depletion of uridine triphosphate (UPT) shortly after galactosamine administration with a resulting accumulation of uridine diphosphate (UDP). They speculated that the decrease in UTP levels interfered with RNA synthesis with subsequent inhibition of the synthesis of proteins and

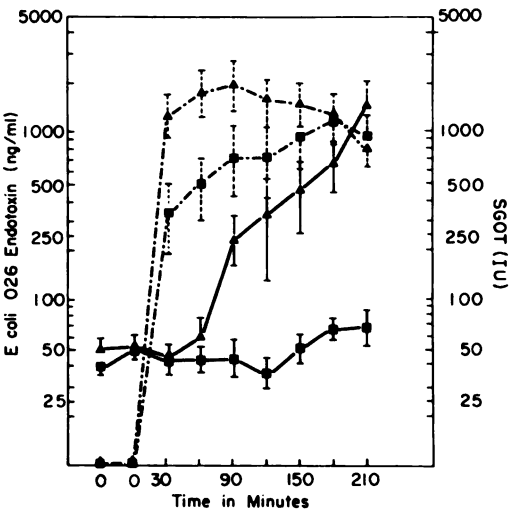

FIG. 2. SGOT activities (——) and serum *E. coli* 026 endotoxin concentration (- · - · -) in rabbits infused with galactosamine (▲) or saline (■). *E. coli* 026 endotoxin levels were significantly higher ($p < 0.05$) in the rabbits receiving galactosamine at 30 and 60 min. SGOT activity was significantly higher in the galactosamine group beyond 90 min.

other macromolecules (1). El-Mofty *et al.* (13) postulated that the changes in level of intracellular UDP-hexosamines were responsible for substantial structural changes in the plasma membrane of the hepatocytes leading to cell necrosis. However, other drugs that induce similar inhibition of protein synthesis, e.g., ethionine (13), cycloheximide (14), and actinonycin D (9), do not cause significant hepatocellular necrosis. This fact as well as the severity of the liver injury suggested alternate explanations for the hepatotoxic effects of galactosamine.

The SGOT activity has been shown to reflect the magnitude of the liver cell injury and/or necrosis after galactosamine administration. Blitzer *et al.* as well as Decker and Keppler (1, 2) have carefully quantitated the liver injury induced by galactosamine using a battery of serum markers such as: SGOT, glutamic pyruvic transaminase, lactate dehydrogenase, sorbitol dehydrogenase, fibrinogen, prothrombin, ammonia, and amino acids (21). They found no histological evidence of injury in other areas rich in SGOT such as the heart and striated muscle. Therefore, in the present study we considered that the SGOT values accurately reflected early hepatocyte injury.

The histological changes observed in the liver after galactosamine administration have been extensively studied (1, 2, 14, 26, 27). The lack of significant histological differences between controls and animals which received galactosamine in our experiments is not surprising since the time frame used in this study was too brief for significant morphological changes to occur. Indeed, Koff *et al.* observed by light microscopy the first signs of histologic damage post-galactosamine administration only after 4 hr (27).

It should be mentioned that evisceration can produce hemodynamic changes not detected by measuring the blood pressure alone. However, in preliminary studies we did not observe significant elevation of SGOT activity 24 hr after resecting the small bowel and colon in rabbits. Furthermore, after evisceration, not only is there a marked reduction in the amount of endogenous endotoxin, but also in the level of splanchnic hormones and metabolic products such as ammonia and bile acids. The role of these factors has not been evaluated in the pathogenesis of galactosamine liver injury.

In the absorption studies we used an immunoradiometric assay for *E. coli* 026 endotoxin rather than the Limulus assay (22–24). This assay is specific for *E. coli* 026 LPS which is not present in the rabbit colon, thus serving as a marker for endotoxin absorption. We observed that endotoxin was readily absorbed from the colon and detected in the blood of the animals injected with galactosamine and the controls injected with saline. The absorption of endotoxin could be due in part to a nonspecific depression of the reticuloendothelial system secondary to anesthesia (25). However, despite the similar serum concentration of LPS in both groups of animals, SGOT elevation was observed only in the animals injected with galactosamine. We believe that galactosamine may render the liver susceptible to the toxic effects of endotoxin. This conclusion is further supported by the first part of our experiment where removal of the colon (resevoir of endotoxin) neutralized the toxicity of galactosamine to the liver. Whether the endotoxemia detected in these animals represents increased absorption, decreased detoxification of LPS by the liver, or a combination of both could not be determined in this study.

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1. Decker K, Keppler D. Galactosamine induced liver injury. In: Popper H, Schaffner F, eds. Progress in Liver Disease. New York, Grune & Stratton, Vol 4:pp183–199, 1977.
2. Decker K, Keppler D. Galactosamine hepatitis: Key role of nucleotide deficiency in the pathogenesis of cell injury and cell death. Rev Physiol Biochem Pharmacol 71:78–106, 1974.
3. Nolan JP. The role of endotoxin in liver injury. Gastroenterology 69:1346–1356, 1975.
4. Nolan JP. Endotoxin reticuloendothelial function and liver injury. Hepatology 1:458–465, 1981.
5. Nolan JP, Ali MV. Endotoxin and the liver. II. Effects of tolerance on carbon tetrachloride induced injury. J Med 4:28–38, 1973.
6. Nolan JP, Leibowitz A. Endotoxin and the liver. III. Modification of acute carbon tetrachloride injury by

polymyxin B—An antiendotoxin. Gastroenterology 75:445–449, 1978.
7. Broitman SA, Gottileb LS, Zamcheck N. Influence of Neomycin and ingested endotoxin in the pathogenesis of choline deficiency cirrhosis in the adult rat. J Exp Med 119:633–641, 1969.
8. Liehr H, Grun M, Seelig HP, Seelig R, Reutter W, Heine WD. On the pathogenesis of galactosamine hepatitis. Virchows Arch B, Cell Pathol 26:331–344, 1968.
9. Grun M, Liehr H, Rasenack U. Significance of endotoxemia in experimental galactosamine hepatitis in the rat. Acta Hepatol Gastroenterol 23:64–81, 1976.
10. Rasenack J, Koch HK, Nowack J, Lesch R, Decker K. Hepatotoxicity of D-galactosamine in the isolate perfused rat liver. Exp Mol Pathol 32:264–275, 1980.
11. Al-Tuwaijri A, Akdamar K, DiLuzio N. Modification of galactosamine induced liver injury in rats by reticuloendothelial system stimulation or depression. Hepatology 1:107–113, 1981.
12. Leibowitz AI, Vladutiu AO, Nolan JP. Immunoradiometric assay of endotoxin in serum. Clin Chem 25:68–70, 1979.
13. El-Mofty SK, Scrutton MC, Farber JL. Early reversal of plasma membrance injury in galactosamine induced liver cell death. Amer J Pathol 79:579–595, 1975.
14. Koff RS, Connelly LJD. Modification of hepatotoxicity D-galactosamine in the rat by Cycloheximide. Proc Soc Exp Biol Med 151:519–522, 1976.
15. Grun M, Liehr H, Rasenack U, Brunswig D. Influence of liver RES on toxic liver damage due to galactosamine. Acta Hepatol Gastroenterol 21:5–15, 1974.
16. Rasenack U, Grun M, Liehr H. Galactosamine hepatitis in rats with different phagocytic activity of liver RES. Acta Hepatol Gastroenterol 22:29–35, 1974.
17. Reutter W, Bauer C, Bachmann W, Lesch R. The galactosamine refractory regenerating liver. In: Lesch R, Reutter W, eds. Liver Regeneration after Experimental Injury. New York, Stratton Intercontinental Book Corp, pp259–272, 1975.
18. Morrison DC, Ulevitch RJ. The effects of bacterial endotoxin on host mediation systems. Amer J Pathol 93:527–617, 1978.
19. Utili R, Abernathy CO, Zimmerman HJ. Endotoxin effects on the liver. Life Sci 20:553–568, 1977.
20. Bradley SG. Cellular and molecular mechanisms of action of bacterial endotoxin. Annu Rev Microbiol 33:67–94, 1979.
21. Blitzer BL, Waggoner JG, Jones EA, Gralnick HR, *et al.* A model for a fulminant hepatic failure in the rabbit. Gastroenterology 74:664–671, 1978.
22. Martinez LAG, Quintiliani R, Tilton R. Clinical experience on the detection of endotoxemia with the limulus test. J Infect Dis 127:102–105, 1973.
23. Munford R. Endotoxins and the liver (editorial). Gastroenterology 72:532–535, 1978.
24. Stumacher RJ, Kovnat M, McCabe W. Limitation of the usefulness of the limulus assay for endotoxin. N Engl J Med 288:1261–1264, 1973.
25. Hershey SG, Altura BM. Function of the reticuloendothelial system in experimental shock and combined injury. Anesthesiology 30:138–142, 1961.
26. Shinozuka H, Farber JL, Konishi Y, Anukarahanonta T. D-Galactosamine in acute liver cell injury. Fed Proc 32:1516–1526, 1973.
27. Koff RS, Gordon G, Sabesin SM. D-Galactosamine hepatitis. I. Hepatocellular injury and fatty liver following a single dose. Proc Soc Exp Biol Med 137:696–701, 1971.

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Failure of Athymic-Nude Mice Sensitized with *Bacillus Calmette–Guérin* to Produce Interferon in Response to Purified Protein Derivative (41556)

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Abstract. Unlike their +/+ and nu/+ littermates, homozygous athymic-nude (nu/nu) mice that had been sensitized with *Bacillus Calmette–Guérin* (BCG) failed to produce interferon (IFN) in response to purified protein derivative (PPD). Likewise, spleen cells derived from BCG-sensitized nu/nu mice failed to produce IFN when exposed to PPD *in vitro*. These findings suggest that the T-cell subset that responds to specific antigens (i.e., PPD) is absent in athymic-nude mice.

The production of immune (or type II) interferon (IFN-γ)[3] has been described in various experimental systems, such as spleen cells induced with the T-cell mitogens concanavalin A (Con A), phytohemagglutinin (PHA), or staphylococcal enterotoxin A (SEA); lymphoid cells treated with anti-lymphocyte serum or galactose oxidase; primary and secondary mixed lymphocyte cultures (MLC); and, finally, sensitized cells upon reexposure to the specific antigen (for a review see (1)).

The induction of IFN-γ depends on the cooperation of different cell types, i.e., T and B lymphocytes and macrophages (2), although the relative contribution of these different cell types in the induction process has not been clearly assessed. Moreover, cloned T cells are by themselves capable of producing IFN-γ when exposed to Con A or specific antigens (3).

Spleen cell cultures derived from athymic-nude (nu/nu) mice produce interferon in response to PHA or SEA (4, 5) but fail to do so when stimulated by either allogeneic cells or Con A (6, 7). It is likely that the subpopulation of T cells that normally responds to Con A or alloantigens is absent in nu/nu mice (or nu/nu mouse spleen cells) (7). This contention may also hold for specific antigens, since, as described in the present report, nu/nu mice which have been immunized with BCG fail to produce interferon in response to PPD under conditions where their heterozygous littermates and homozygous +/+ counterparts are able to mount considerable interferon yields.

1. Materials and Methods. *1.1 Mice.* Outbred NMRI (Naval Medical Research Institute) nu/nu, nu/+, and +/+ mice were reared and maintained as described before (8, 9). C57Bl/6J BOM nu/nu, nu/+, and +/+ mice were obtained from G-I Bomholtgård Ltd., Ry, Denmark. Mice weighing 20–25 g were used throughout all experiments.

1.2 Lymphoproliferation assay. Unfractionated spleen cells were suspended at 5×10^5 cells/ml in RPMI 1640 medium, supplemented with 5% FCS (fetal calf serum), antibiotics, and 5×10^{-5} *M* 2-mercaptoethanol. The cells were plated in flat-bottomed microplates (Sterilin) in 200-μl volumes. To activate the lymphocytes, 20 μl of freshly prepared mitogen solutions were added per well. Final concentrations of the mitogens were: Con A (concanavalin A, Pharmacia), 4 μg/ml; PHA (phytohemagglutinin, Wellcome), 15 μg/ml, and LPS (lipopolysaccharide *E. coli* 055B5, Instituut Pasteur Brabant), 20 μg/ml. The cells were cultured for 72 hr in a humidified CO_2 incubator, pulse labeled with 0.4

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[3] Abbreviations used: BCG, *Bacillus Calmette-Guérin*; PPD, purified protein derivative; PHA, phytohemagglutinin; Con A, concanavalin A; SEA, staphyloccocal enterotoxin A; MLC, mixed lymphocyte culture; VSV, vesicular stomatitis virus; NDV, Newcastle disease virus; MuIFN-α,β, murine type I interferon; MuIFN-γ, murine type II (immune) interferon; PBS, phosphate-buffered saline.

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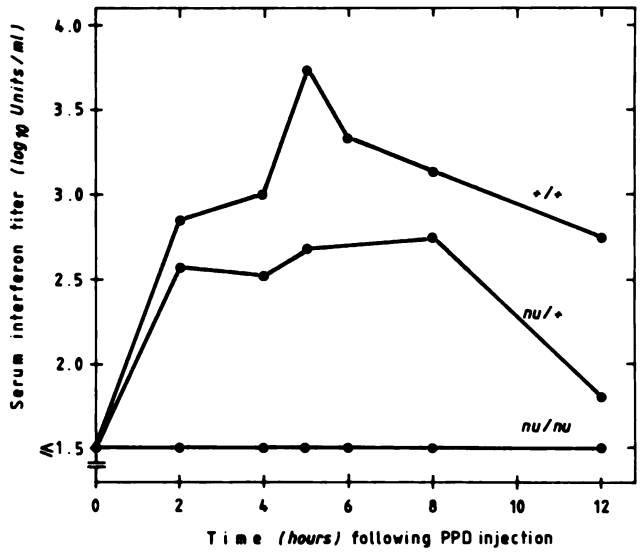

FIG. 1. Serum interferon titers of NMRI nu/nu, nu/+, and +/+ mice immunized with BCG and challenged 2 weeks later with PPD. The interferon titers were determined at different times after PPD injection on the pooled serum fractions of three mice and are expressed in laboratory MuIFN-γ units; 0.6 log unit should be added to each value to obtain the interferon titers in terms of the National Institute of Health (USA) MuIFN-α, β standard G002-904-511.

μCi [*methyl*-^{3}H]thymidine (Amersham, 8.3 mCi/mg) per well and harvested 5 hr later on a Titertek Cell Harvester (Skatron). The radioactivity recovered from the filters was measured in a Beckman LS.230 liquid scintillation counter. All experiments were done in quadruplate. Results are expressed as the mean ± SD counts per minute (cpm).

1.3. Interferon production. 1.3.1. Antigen-induced IFN. The mice were sensitized intravenously with BCG (BCG strain GL 2, grown as surface pellicle) at 5×10^6 bacteria/mg/0.2 ml/mouse (or PBS (phoshate-buffered saline) at 0.2 ml/mouse) and 2 weeks later injected intravenously with PPD at 1 mg/0.2 ml/mouse (or PBS at 0.2 ml/mouse). Blood samples were taken at several times (2, 4, 5, 6, 8, and 12 hr after the injection of PPD (or PBS) and the sera were pooled (three mice/group).

For the *in vitro* production of IFN-γ, mice were killed by cervical dislocation 2 weeks after the BCG inoculation. Spleens were removed aseptically, and the cells were dispersed in a loosely fitting Dounce homogenizer, counted in a Coulter counter, and suspended at 5×10^6 cells/ml in RPMI 1640 medium supplemented with 10% FCS, antibiotics, and 5×10^{-5} *M* 2-mercaptoethanol. PPD was added at various doses and aliquots of the culture supernatant were harvested for IFN titration at either Day 1, 2, 3, or 4.

1.3.2. Mitogen-induced IFN. Spleen cells derived from untreated mice were cultured as described in section 1.3.1. The cells were stimulated by Con A (4 μl/ml) or PHA (15 μl/ml) and culture supernatants were harvested for IFN titration on Days 1, 2, 3, and 4.

1.4. Interferon assay. Serum interferon titers were determined by a virus plaque reduction assay in mouse L-929 cells using VSV as the challenge virus. A laboratory standard of mouse type I interferon (MuIFN-α,β), calibrated against the National Institute of Health (USA) mouse type I interferon standard G002-904-511, was included in the interferon assays and gave a titer ($10^{2.9}$ units/ml) that was 0.6 × log unit lower than the expected titer ($10^{3.5}$ units/ml).

2. Results. *2.1. In vivo production of antigen-induced IFN.* As shown in Fig. 1, NMRI +/+ mice which had been immunized with BCG produced significant amounts of MuIFN-γ after PPD injection (up to about 5000 units/ml at the peak of the interferon

response). Their homozygous nu/nu counterparts were completely unresponsive to the PPD challenge. In the heterozygous nu/+ mice intermediate interferon levels were obtained. This indicates that the production of PPD-induced interferon (in BCG-sensitized mice) is under the control of a codominant gene, perhaps expressed only in mature T cells.

The kinetics of appearance of MuIFN-γ in the +/+ mice was similar to that described by Stinebring and Absher (10): thus, the serum interferon titer reached its peak value at 4 to 6 hr after PPD injection and declined to about 10% of its peak value at 12 hr after PPD injection. The data presented in Fig. 1 concern the interferon titers obtained upon a double course of events (BCG infection followed 2 weeks later by a PPD injection). No interferon was detected in the bloodstream of +/+, nu/+, or nu/nu mice which were given BCG followed by PBS or PBS followed by PPD.

2.2 In vitro production of antigen-induced IFN. As shown in Fig. 2, spleen cells from C57Bl/6J +/+ mice, previously immunized with BCG, produced significant amounts of interferon following *in vitro* exposure to PPD. The highest IFN titers were obtained with PPD at 150 and 500 μg/ml (the highest concentrations tested) but even at lower PPD concentrations significant IFN levels were noted. In keeping with the data obtained *in vivo* (section 3.1), homozygous nu/nu spleen cells were totally refractory to the interferon inducing effects of PPD. The heterozygous nu/+ spleen cells produced intermediate interferon levels. Spleen cells derived from nonimmunized +/+ mice did not produce IFN in response to PPD; neither did spleen cells from BCG-immunized mice in the absence of PPD (data not shown).

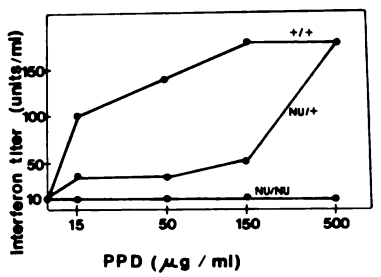

FIG. 2. Interferon titers obtained in the supernatant of spleen cell cultures derived from C57Bl/6J BOM nu/nu, nu/+, and +/+ mice immunized with BCG and exposed to PPD *in vitro*. The spleen cells were removed 2 weeks after immunization of the mice with BCG and incubated with PPD for 24 hr. The interferon titers were determined as a function of the PPD concentration and are expressed in laboratory MuIFN-γ units as explained in the legend to Fig. 1.

TABLE I. INTERFERON PRODUCTION IN SPLEEN CELL CULTURES DERIVED FROM NMRI nu/nu AND +/+ MICE

Inducer[a]	Cell source[b]	Interferon titer[c] 24 hr	48 hr	72 hr	96 hr
PPD	+/+	178	178	178	178
	nu/nu	<10	<10	<10	<10
Con A	+/+	100	56	18	18
	nu/nu	<10	<10	<10	<10
PHA	+/+	178	100	56	18
	nu/nu	<10	<10	<10	<10
NDV	+/+	178	178	178	178
	nu/nu	18	32	32	25

[a] PPD, 500 μg/ml; Con A, 4 μg/ml; PHA, 40 μg/ml; and NDV 100 hemagglutination units/ml.
[b] Used at 5×10^6 spleen cells/ml (derived from mice sensitized with BCG 2 weeks before PPD challenge; not sensitized if used with Con A, PHA or NDV as challenge).
[c] Expressed in laboratory units (see legend to Fig. 1).

2.3 Reactivity of nu/nu mouse spleen cells to T-cell mitogens in vitro. Table I presents the kinetics of the IFN production in cultures of 5×10^6 spleen cells/ml activated with PPD, Con A, PHA, or NDV (Newcastle Disease virus (strain La Sota), inactivated by β-propiolactone). BCG-sensitized +/+ spleen cells produced substantial amounts of interferon in response to PPD. This interferon production remained constant for at least 4 days. However, BCG-sensitized nu/nu spleen cells failed to produce IFN in response to PPD, irrespective of the time of incubation (1, 2, 3, or 4 days).

Although other authors have reported that PHA is capable of inducing interferon in nude mice (4), we were unable to demonstrate interferon induction by PHA in nu/nu mouse spleen cells, whether these cells were derived from NMRI nu/nu mice (Table I) or C57Bl/6J BOM nu/nu mice (data not shown). Con A also failed to induce IFN in nu/nu mouse spleen cells, which is consistent with the observations of Kirchner *et al.* (7). In contrast with the nu/nu mouse spleen cells, nu/+ and +/+ mouse spleen cells produced significant

TABLE II. PROLIFERATION OF SPLEEN CELLS DERIVED FROM nu/nu, nu/+, AND +/+ MICE

	Inducer				
e	None[a,c]	PPD[a,b]	Con A[a,c]	PHA[a,c]	LPS[a,c]
1	1570 ± 244	20,705 ± 451	1,068 ± 170	385 ± 28	31,995 ± 2350
	1530 ± 106	18,470 ± 798	69,330 ± 2595	32,295 ± 2036	65,715 ± 3775
	1510 ± 133	8,830 ± 410	107,615 ± 6805	56,725 ± 4020	69,740 ± 3140

he values are expressed as the mean ± SD radioactivity counts (cpm) obtained for quadruplate cell cultures.
pleen cells derived from C57Bl/6J BOM mice, sensitized 2 weeks earlier with BCG and incubated *in vitro* with (500 μg/ml) for 24 hr; [*methyl*-^{3}H]thymidine was added during the last 5 hr of this 24-hr incubation period.
pleen cells derived from NMRI mice and stimulated *in vitro* with Con A, PHA, or LPS as described in section *methyl*-^{3}H]thymidine was added during the final 5 hr of the 3-day incubation period.

unts of interferon in response to either A or PHA. The IFN titers induced by A and PHA reached their peak value at ır and declined during the next 3 days. ıe IFN levels were comparable to those ribed in the literature.

lthough unresponsive to the interferon-cing activity of PPD, Con A, and PHA, ıu mouse spleen cells produced appreciamounts of IFN upon exposure to NDV. ever, these IFN yields were significantly r than those obtained with nu/+ or +/+ se spleen cells.

s shown in Table II, nu/nu mouse spleen did not proliferate upon stimulation with T-cell mitogens Con A and PHA. The B-mitogen LPS caused a significant prolifve response in nu/nu mouse spleen cell ures, albeit to a lesser extent than in nu/ ıd +/+ spleen cell cultures. It is notehy that BCG-sensitized nu/nu spleen cells ved a more pronounced proliferative re-ın to PPD than the corresponding wild-+/+ spleen cells. The diminished +/+ n cell response may possibly be related ıe growth-inhibitory effect of the inter-ı produced by these cultures.

4 Characterization of the antigen- and gen-induced interferons. The interferons were produced by BCG-immunized +/ ıice and +/+ mouse spleen cells in re-se to PPD were characterized as authen-MuIFN-γ by their sensitivity to pH 2.0 ıplete loss of interferon activity after di-s against 0.1 *M* glycine–HCl, pH 2.0, for r), relative resistance to heating at 56° for nin (only 0.5 log decrease in interferon ity), neutralization by specific antibodies ed against SEA-induced MuIFN-γ (11)), lack of neutralization by anti-MuIFN-α,β serum. Mitogen-induced IFN was only partially destroyed by pH 2.0 treatment (residual interferon activity 20 to 30%). It was neutralized by specific antibodies to MuIFN-γ, but not by antibodies to MuIFN-α,β.

3. Discussion. Whereas PHA (4) and SEA (5) are capable of inducing IFN-γ in nu/nu mouse spleen cell cultures, Con A and alloantigens are not able to do so (6, 7). A failure to induce IFN-γ in athymic-nude mice has also been noted with the bacterial immunopotentiator OK-432 (12). As further documented in the present paper, specific antigens, i.e., PPD, also fail to induce IFN-γ in BCG-sensitized nu/nu mice.

Recent studies of mitogen-induced DNA synthesis and interferon production in cells from different lymphoid tissues have indicated that certain mitogens such as PHA, SEA, and Con A selectively activate T-cell subpopulations (13). In view of the present data on interferon induction by specific antigens in nude mice and nude mouse spleen cells and the previously published data on interferon induction by PHA, SEA, Con A, and alloantigens in nude mouse spleen cells, one may postulate that the T-cell subset(s) responding to PHA and SEA are present in nude mouse spleen, whereas the subset(s) responsive to Con A, alloantigens, or specific antigens are not. However, some caution is warranted in the interpretation of the aforementioned data. Indeed, IFN-γ preparations obtained with SEA or PHA as the inducer may also contain IFN-α (14) and Con A-induced IFN-γ may be contaminated by IFN-β (15). Furthermore, the contention that nude mouse spleen cells are able to produce IFN in response to PHA (4) should be reevaluated since we were unable to obtain IFN production with PHA in spleen

cell cultures derived from nude mice of different genetic background (NRMI and C57Bl/6J).

The patterns of production of type I and type II interferon are clearly differentiated. While unresponsive to specific antigens (i.e., PPD), nude mice produce substantial amounts of interferon in response to type I interferon inducers, such as NDV (this report) and polyinosinic acid · polycytidylic acid (9). Moreover, macrophages derived from nude mice infected with BCG or *Corynebacterium parvum* produce similar type I interferon levels as macrophages derived from their normal littermates (16).

Although antibodies raised against SEA-induced interferon can neutralize different immune interferons (induced by PHA, Con A, MLC, and specific antigens) (5), this does not necessarily imply that they are all identical. The diversity of the induction systems and the heterogeneity observed upon purification (17) could indicate that they are not. For human leukocyte interferon there exist at least 12 different genes (18) and the products of these genes show important functional differences, for example in host-range (species specificity). A similar situation may also occur with IFN-γ.

We thank Dr. H. Heremans for the generous gift of anti-MuIFN-γ antiserum, Dr. J. Nyabenda for the PPD, and K. Palfliet for excellent technical assistance.

1. Torrence PF, De Clercq E. Inducers and induction of interferons. In: Pharmacology and Therapeutics. New York, Pergamon Press, Part A, Vol 2, No. 1, pp. 1–88, 1977.
2. Sonnenfeld G, Mandel AD, Merigan TC. In vitro production and cellular origin of murine type II interferon. Immunology **36**:883–890, 1979.
3. Marcucci F, Waller M, Kirchner H, Krammer P. Production of immune interferon by murine T-cell clones from long-term cultures. Nature (London) **291**:79–81, 1980.
4. Wietzerbin J, Stefanos S, Falcoff R, Lucero M, Catinot L, Falcoff E. Immune interferon induced by phytohemagglutinin in nude mouse spleen cells. Infect Immun **21**:966–972, 1978.
5. Osborne LC, Georgiades JA, Johnson HM. Classification of interferon with antibody to immune interferon. Cell Immunol **53**:65–70, 1980.
6. Kirchner H, Zawatzky R, Engler H, Schirrmacher V., Becker H, von Wussow P. Production of interferon in the murine mixed lymphocyte culture. II Interferon production is a T cell-dependent function, independent of proliferation. Eur J Immunol **9**:824–826, 1979.
7. Kirchner H, Fenkl H, Zawatzky R, Engler H, Becker H. Dissociation between interferon production induced by phytohemagglutinin and Concanavalin A in spleen cell cultures. Eur J Immunol **10**:224–225, 1980.
8. De Clercq E. Tumor induction by Moloney Sarcoma Virus in athymic nude mice. J Natl Cancer Inst **54**:473–477, 1975.
9. De Clercq E. Effect of mouse interferon and poly(I) · poly(C) on L-cell tumor growth in nude mice. Cancer Res **37**:1502–1506, 1977.
10. Stinebring WR, Absher PM. Production of interferon following an immune response. Ann NY Acad Sci **173**:714–718, 1970.
11. Heremans H, De Ley M, Billiau A, De Somer P. Mouse immune interferon: Production and partial purification. In: De Maeyer E, Galasso G, Schellekens H, eds. The Biology of the Interferon System. Amsterdam, Elsevier/North-Holland, pp 295–299, 1981.
12. Matsubara S, Suzuki F, Ishida N. Induction of immune interferon in mice treated with bacterial immunopotentiator, OK-432. Cancer Immunol Immunother **6**:41–45, 1979.
13. Archer DL, Smith BG, Ulrich JT, Johnson HM. Immune interferon induction by T-cell mitogen involves different T-cell subpopulations. Cell Immunol **48**:420–426, 1979.
14. Wiranowska-Stewart M. Heterogeneity of human gamma interferon preparations: Evidence for presence of alpha interferon. J Interferon Res **1**:315–321, 1981.
15. De Ley M, Van Damme J, Claeys H, Weening H, Heine JW, Billiau A, Vermijlen C, De Somer P. Interferon induced in human leukocytes by mitogens: Production, partial purification and characterization. Eur J Immunol **10**:877–883, 1980.
16. Neumann C, Macher E, Sorg C. Interferon production by *Corynebacterium parvum* and BCG-activated murine spleen macrophages. Immunobiology **157**:12–23, 1980.
17. Stefanos S, Wietzerbin J, Huygen K, Falcoff E. Characterization and partial purification of antigen-induced mouse γ interferon. In: De Maeyer E, Galasso G, Schellekens H, eds. The Biology of the Interferon System. Amsterdam, Elsevier/North-Holland, pp 285–288, 1981.
18. Nagata S, Mantei N, Weissman C. The structure of one of the eight or more distinct chromosomal genes for human interferon-α. Nature (London) **287**:401–416, 1980.

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ABSTRACTS

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LIPOPROTEIN LIPASE ACTIVITY IN SEDENTARY AND ACTIVE RATS. B.W. Bennett, G.J. Bagby, J. Johnson, J.R. Porter, R.E. Shepherd. Dept. of Physiology, Louisiana State Univ. Med. Ctr., New Orleans, LA. 70112.

The objective of this study was to determine the effect of 6 wks of voluntary physical activity by rats on lipoprotein lipase activity (LPLA) in heart, epididymal fat pad, soleus, and red vastus muscle. Throughout the study the animal facility was luminated from 0600 h to 1800 h. Active rats (running in individual exercise wheels) averaged 10 km/day during the last 3 wks of the study. Rats were killed by decapitation near the peak (2000 h) and valley (0800 h) of their corticosterone diurnal cycle. Adrenal corticosterone was similar in sedentary and active rats with the content increased significantly at 2000 h compared to 0800 h. Tissue LPLA did not differ between the two time periods examined in either sedentary or active rats. In active rats, red vastus muscle was the only tissue that consistently exhibited an increased LPLA when compared to sedentary rats. Adipose tissue LPLA per fat pad decreased in active rats. The results indicate that 6 wks of voluntary exercise in the rat produced tissue dependent changes in LPLA. (Supported by NIH grants AM 28521 and HL 04795.)

MECAMYLAMINE (MA) ANTAGONIZES POTENTIATION OF SODIUM PENTOBARBITAL (NAP) INDUCED SLEEP BY NICOTINE. David T. Butsch, Benjamin E. Alderete, and Arvind T. Modak. Dept. of Pharmacology, UTHSCSA, 7703 Floyd Curl Drive, San Antonio, Tx 78284.

We have shown that nicotine exhibits selectivity in modifying the hypnotic effects of CNS depressants in mice. The purposes of this study were to establish the dose response for nicotine and to determine the mechanism(s) of action of nicotine. Sprague Dawley male ICR mice were used. Sleep time was determined by taking the difference between the time of loss and regaining of the righting reflex. Intraperitoneal (IP) injections of 5 ml/kg normal saline or 2.5 or 5 mg/kg nicotine were given 15 minutes prior to 50 mg/kg NAP, I.P. In another study mice were given 0.5 mg/kg MA, I.P., 15 minutes prior to nicotine. Nicotine, was administered I.P. 15 minutes prior to 50 mg/kg NAP, I.P. Sleep times in min ± S.D. (number of mice) for sodium pentobarbital were: control: 48 ± 8.2 (15), 2.5 mg nicotine: 60.2 ± 9.2 (14), 5 mg nicotine: 72.9 ± 11.7 (15), 0.5 mg M.A. plus 2.5 mg/kg nicotine: 43.0 ± 1.5 (15). These data show that nicotine potentiated NAP induced sleep time in a dose dependent manner. Also, MA, a nicotine antagonist, normalized NAP induced sleep time in mice pretreated with nicotine. These data indicate that the nicotinic receptors are involved in nicotine potentiation of sodium pentobarbital induced sleep. (Supported by Grant 5SO6RR08170-05).

DOG HEPATIC LIPASE: A TRI-FUNCTIONAL ENZYME. Mei-Yu Chuang, Laurence Wong, and Paul S. Roheim. Department of Physiology, Louisiana State University Medical Center, 1542 Tulane Avenue, New Orleans, Louisiana 70112

Hepatic lipase can be found in the liver and post-heparin plasma. Its physiological role in lipoprotein metabolism is not clear. It may be a tri-functional enzyme consisting of tri-, mono-acylglycerol lipase and phospholipase A_1 activities. We are using a monoclonal antibody immunoaffinity chromatography to characterize this enzyme. The immunoaffinity column shows that the antibody is specific to dog hepatic lipase and does not cross-react with lipoprotein lipase. When a preparation of dog hepatic lipase is passed through this column, all three enzyme activities decrease to the same extent and all three enzyme activities can be found to associate with the column. The lipase has native molecular weight of greater than 200,000. SDS-Polyacrylamide gel electrophoresis reveals that one major protein band (MW=84,000) and two minor protein bands (MW=58,000 and 52,000) can be concentrated from dog post-heparin plasma by this column. We conclude that tri-, mono-acylglycerol lipase and phospholipase A_1 activities of dog hepatic lipase is on the same molecule. (Supported by NIH Program Project #HL25596).

PROTEIN SYNTHESIS IN PULMONARY MACROPHAGES: EFFECT OF PHAGOCYTOSIS. Dominic Fan; Dept. of Pharmacology, LSU Medical Center, New Orleans LA 70112. Donald Massaro; Dept. of Medicine, UM Medical Center and the VA Hospital Miami FL 33101.

Extracellular and intracellular free and tRNA-bound phenylalanine was determined in pulmonary macrophages in culture. Phagocytosis resulted in an increased rate of protein synthesis irrespective of which precursor pool was used in calculation. The rate of protein synthesis was increased per ug of polyribosomal RNA; but there was no increase in the amount of polyribosomal RNA in phagocytizing macrophages The increase in the rate of protein synthesis (1 4 fold) was almost identical to the increase (1.3 fold) in the rate of ribosome transit (the time required for a ribosome after having become attached to a messenger RNA, to complete translation and release a finished polypeptide) in phagocytizing macrophages. The decreased ribosome transit time during phagocytosis occurred without a change in the average molecular weight of macrophage proteins Therefore phagocytosis increases the rate of protein synthesis in attached pulmonary macrophages and this increase can be accounted for almost completely by an increased rate of polypeptide chain elongation and/or termination. (Supported in part by NHLI Training Grant HL07283, NHLI Research Grant HL20366 and the VA Hospital.)

EFFECTS OF SEXUAL MATURATION ON TESTOSTERONE PRODUCTION BY MOUSE TESTES IN VITRO. David Gonzalez & Andrzej Bartke. Department of OB/GYN, University of Texas Health Science Center, San Antonio, TX, 78284.

During puberty, production of testosterone (T) by the testis greatly increases, but the mechanisms responsible for this are not completely understood. We have examined production of T in vitro by testes from immature mice (26, 37 & 46 days old) and adult mice (over 60 days old) in the presence of 0, 3.1, 6.2, and 12.5 mIU/ml human chorionic gonadotropin (hCG), a hormone with luteinizing hormone (LH) activity. These testes were incubated for 4 hours and T concentrations were measured by radioimmunoassay (RIA). Compared to the testes of adult mice, testes from the 27-day-old mice produced little T (106 ± 20 vs 289 ± 19 pg/mg) and showed a very poor response to hCG (with 6.2 mIU/ml, 805 ± 160 vs 6157 ± 1057 pg/mg). In contrast, testes from 37-day-old mice produced more T than did testes of the adult mice, both with and without hCG. Testes from the 46-day-old mice showed little differences from the adult. These results appear to indicate that responsiveness of the testis to LH increases greatly during the early stages of sexual maturation (between 26-37 days). This increase in responsiveness of T production could be due to an increase in the concentration of LH (hCG) receptor sites, which are located on the Leydig cells of the testis. Supported by HD 12642.

ARACHIDONIC ACID DEPRESSION OF THE ISOLATED PERFUSED RAT HEART. Gary S.Hambrecht and James G. Hilton. Departments of Pharmacology and Toxicology, University of Texas Medical Branch and Shriners Burns Institute, Galveston, Texas 77550.

These studies were carried out on the isolated rat heart perfused with Ringer's Locke solution at 37 5° C Isometric force of contraction was recorded by use of a Grass FT03 Force Transducer. Observations were recorded every 5 minutes for 120 minutes. Arachidonic acid (0.5 mM) was infused for a period of 10 minutes at a rate of 1 ml/min beginning 15 minutes after perfusion was started. A significant decrease in force of contraction occurred during the infusion of arachidonic acid. This decrease in force of contraction continued after infusion ceased. Allen and Levi JPET 214: 45-59 1980) have proposed that the decrease in force of contraction following arachidonic acid was due to the formation of prostaglandins and have demonstrated that indomethacin will prevent the decrease. With the dose of arachidonic acid used in this study indomethacin did not prevent the decrease in force of contraction observed during infusion but did prevent the continuing decrement of activity observed after infusion was stopped. These results suggest that there are at least two components to the arachidonic acid decrease in cardiac force of contraction. An early response to arachidonic acid which appears to be independent of prostaglandin formation and a latter continuing response which is dependent upon cyclo-oxygenase activity.

ENDOTOXIN INDUCED ALTERATIONS IN GLYCEROL REMOVAL IN DOGS. O.P. McGuinness and J.J. Spitzer. Dept. of Physiology, LSU Medical Center, New Orleans, LA 70112

The metabolic clearance rate (MCR) of glycerol (gly) was investigated in the anesthetized dog following the administration of saline (n=7) or E. Coli endotoxin (0.3 mg/kg; n=6). A stepwise infusion of gly was used (4.3,8.7,17.4 mmoles/kg/min) during the early (15-135 min) and late (260-380 min) post-treatment time periods. A steady state arterial gly concentration was obtained within 40 minutes at each infusion rate. The MCR was calculated as the inverse of the slope of the linear relationship between gly infusion rate and the steady state arterial gly concentration. In the saline treated group the MCR was the same during both time periods (early: 316 $\pm$ 0.3, late: 31.6 $\pm$ 0.2 ml/kg/min). The MCR following endotoxin administration was decreased markedly as compared to saline treatment (early: 20.8 $\pm$ 0.3, late: 19.2 $\pm$ 0.3 ml/kg/min). Since the liver is the main site of gly removal preliminary experiments were performed to determine hepatic gly uptake. Calculated hepatic clearance of gly was decreased, corroborating the MCR changes. In conclusion, these results indicate that the marked rise in arterial gly concentration following endotoxin administration is in part due to decreased MCR. The liver appears to be an important site of the endotoxin induced alterations in gly turnover. (Supported by USPH NIH Grant HL 07098.)

TISSUE EXTRACT AND PLASMA CONCENTRATIONS OF IMMUNOREACTIVE GASTRIN IN CHICKENS. D. McKay, K. Inoue, P. Chowdhury, L. Neldon, E. Harper, and P. L. Rayford, Dept. of Physiology-Biophysics, Univ. Arkansas for Med. Sciences, Little Rock, AR 72205.

This study was conducted to measure concentrations of immunoreactive gastrin (IRGa) in tissue extracts and plasma of chickens. Methods: Six chickens were sacrificed and tissue specimens were collected from various organs. In 5 conscious chickens, blood samples were collected before, during and after a 60 min i.v. infusion of bombesin (BBS, 0.6 µg/kg-hr or 3.0 µg/kg-hr). Saline was infused as a control. IRGa levels were measured by radioimmunoassay. Results: IGRa (pmol/g) was found in the cerebral cortex (12.9±1.7), cerebellum (7.9±1.3), proventriculus (1.3±0.8), gizzard (3.5±2.5), duodenum (2.1±0.8), jejunum (1.7±0.3) ileum (6.5±1.0) and colon (0.45±0.20). Plasma IRGa did not change with saline or with 0.6 µg/kg-hr of BBS. With 3 µg/kg-hr of BBS, IGRa in plasma increased from basal levels of 10±1 pmol/L to 14±1 pmol/L at 15 min ($p<0.05$), with an integrated response of 145±62 pmol-60 min/L ($p<0.05$). Conclusions: In contrast to mammals, concentrations of IGRa in chickens were higher in the brain than that found in the gut. Plasma immunoreactive gastrin levels were elevated by 3.0, but not by 0.6, µg/kg-hr of BBS suggesting that the response to BBS may be dose related. Supported by NIH Grant AM30415-01.

EFFECT OF CADMIUM ON PULMONARY FUNCTION IN RATS. R. A. McKnight, Parimal Chowdhury, Loyce Neldon and P. L. Rayford. Dept. of Physiology, Univ. Ark. Med. Sci., Little Rock, AR 72205.

We studied lung function in rats after an intraperitoneal exposure of cadmium. Method: Twelve rats divided into 2 equal groups. Control group received saline while the exposed group received an i.p. injection of $CdCl_2$ (4 mg Cd/kg). Four hrs after the injection animals were anesthetized, femoral artery and vein exposed and a tracheal cannula was attached to a two way valve and simple water manometer in order to collect expired air and induce an expiratory load pressure. The cannula was also attached to a transducer to monitor inspiratory and expiratory tracheal pressure. Pressure differences were measured at different times under expiratory load pressure, then measurements were repeated in rats with open chests. Results: Pressure differences (ΔP) as a function of expiratory load pressure was significantly lower in Cd-exposed animals than that of control. Static deflation volume obtained per gm of dry lung weight as a function of pulmonary pressure indicated a significant increase in lung volumes at 20, 25 and 30 mm of pressure in Cd-exposed animals. Blood and expired air parameter measurements indicated no changes in control and exposed animals. Conclusion: Cd exposed rats underwent pulmonary injury resulting in the changes in their lung function. (Supported by NIH Grant AM30415-01)

BRAIN PHOSPHOPROTEIN PHOSPHATASES: CHARACTERIZATION USING A SYNTHETIC PHOSPHOPEPTIDE AS SUBSTRATE. K.E. Vrana and R. Roskoski, Jr. (SPON: J.R. Porter) Department of Biochemistry, LSU Medical Center, New Orleans, LA 70119.

Phosphoprotein phosphatases are a broad class of enzymes which catalyze the dephosphorylation of a wide variety of phosphoproteins. To date, assay procedures have utilized a number of radiolabelled phosphoproteins as substrates which possess heterogeneous sites of phosphorylation (e.g., phosphohistone, phosphocasein and phosphoprotamine). A new assay procedure has been developed which utilizes a synthetic heptapeptide (leu-arg-arg-ala-ser-leu-gly) phosphorylated at the serine residue as a substrate. In contrast to the phosphoproteins, this peptide contains a single, phosphorylated serine in a specified sequence and permits the preparation of substrate solutions of known concentration. Following incubation with phosphatase activity, the phosphopeptide is adsorbed to phosphocellulose paper and the liberated, radioactive phosphate is resolved by eluting into a dilute phosphoric acid solution. The radioactivity is quantitated by measuring Cerenkov emission. Using this synthetic phosphopeptide, three distinct phosphoprotein phosphatase activities have been identified in the DEAE-cellulose elution profiles of bovine brain homogenates. (Supported by USPHS Grants NS-15994 and T32HL07098)

SOFT-AGAR TUMOR STEM CELL ASSAYS: EFFECT OF OXYGEN. Dominic Fan; LSU Medical Center, New Orleans, LA 70112. Carol Swinkin and Awtar Krishan; Comprehensive Cancer Center for the State of Florida, UM Medical Center, Miami, FL 33101.

Mouse leukemic L1210 cells and xenografts of a human melanoma and a cervical carcinoma were grown under various concentrations of O_2 in the soft-agar assay. In L1210 cells, plating efficiency (PE) was higher under hypoxic conditions than the conventional 20% O_2 atmosphere. Cytotoxic effects (CE), as determined by the number of colonies surviving drug exposure were more pronounced under 20% than 5% or 0% O_2. In melanoma cells, PE was highest under 5% O_2. CE of Adriamycin (ADR), Cis-platinum (Cis-Pt) and Vinblastine (VLB) were higher in 5% than 20% O_2. CE of Bleomycin (BLM), Cis-Pt and VLB were also higher in 5% than 0% O_2. Low concentrations of Cis-Pt and VLB stimulated PE under 20% O_2. In cervical carcinoma cells, PE and colony size were increased in 5% O_2. CE of ADR, BLM and Cis-Pt (1-10 ug/ml) were increased in higher O_2 concentrations. In contrast, CE were higher in cells exposed to Cis-Pt (0.1 ug/ml) and VLB (0.001-0.01 ug/ml) under 5% O_2. At low concentrations, BLM, Cis-Pt and VLB promoted growth of cells under 0% O_2. Thus O_2 had great influence on the outcome of the soft-agar assay, indicating the need for caution in interpretation of chemosensitivity data from in vitro studies for clinical use. (Supported in part by PHS G CA-29360 by the NCI, DHSS.)

TROPHIC EFFECT OF TRYPSIN INHIBITOR ON THE REMNANT PANCREAS AFTER MAJOR PANCREATECTOMY IN RATS. Nobuo Baba, T. Suzuki, T. Tobe. First Department of Surgery, Kyoto University Medical School, Kyoto, Japan.

The effect of trypsin inhibitor was studied in rats after 85% pancreatectomy. Method: Pancreatectomy was performed on male rats. Rats were given synthetic trypsin inhibitor (0.5%, FOY305) via drinking water. Endocrine functions of pancreas were examined by intravenous administration of glucose (0.5 g/kg b.w.) and insulin content in the pancreas. Exocrine functions of pancreas were examined by PFD-test (dosing of PT-BAPA 30 mg/kg b.w.) and protein and enzyme contents in the pancreas. Results: Impairments of the glucose elimination rate and the integrated insulin response after major pancreatectomy were improved with trypsin inhibitor for 4 and 12 wks. Trypsin inhibitor administered for 13 wks caused a 1.3 fold increase in pancreatic insulin content. The PFD-test was improved in rats treated with trypsin inhibitor at 4 and 12 wks. The pancreatic amylase, lipase, and protein contents were 1.9, 1.7, and 2.1-fold greater than control. Conclusion: We conclude that oral administration of trypsin inhibitor is effective in improving pancreatic endocrine and exocrine functions after major pancreatectomy in rats.

LEVELS OF CHOLECYSTOKININ, PANCREATIC POLYPEPTIDE AND GASTRIN IN DOGS FOR EIGHT HRS AFTER FOOD. K. Inoue, D. McKay, E. Harper, L. Neldon, P. Chowdhury, and P. L. Rayford. Univ. Ark. Med. Sci., Little Rock, AR 72205.

We have measured postprandial levels of gastrin, cholecystokinin (CCK) and pancreatic polypeptide (PP) in dogs for eight hrs. Materials and Methods: Thirteen dogs were fasted for 24 hrs before being allowed to eat 400 g of standard dog food mixed with 100 ml of milk. Peripheral blood samples were collected before and at regular intervals for 8 hrs after food for radioimmunoassay measurement of gastrin, CCK and PP. Results: Basal levels of gastrin were 9±1 pmol/L and increased significantly to 30±4 pmol/L at 5 min after food: they remained significantly elevated throughout the study. PP was 46±6 pmol/L at basal, 1605±670 pmol/L at 5 min, 586±75 pmol/L at 15 min and was significantly elevated throughout the study. Basal CCK was 10.7±1.3 pmol/L and increased significantly to 12.2±1.7 pmol/L at 30 min: CCK responses to food was triphasic. The integrated responses of gastrin, CCK and PP were 5.9±0.8, 1.2±0.3, 217.7±35.5 nmol - 480 min/L, respectively. Conclusion: The response of gastrin and PP to food was immediate whereas responses of CCK occurred later. All responses were prolonged with gastrin being uniphasic; PP biphasic and CCK triphasic. The results suggest that in dogs food is a potent and prolonged stimulator of gastrin, CCK and PP. (Supported by NIH Grant AM30415-01)

EFFECT OF GASTRIN-RELEASING PEPTIDE ON SERUM INSULIN LEVELS IN ADULT MALE RATS. George H. Greeley, Jr., James C. Thompson. Department of Surgery, The University of Texas Medical Branch, Galveston, TX 77550

The purpose of this study was to examine the action of gastrin-releasing peptide (GRP) on serum insulin levels in male rats. Methods. GRP (10 µg) or saline (controls) was given intravenously under light ether anesthesia. Serum was collected before, and 10, 30, 60, and 120 minutes after GRP or saline (controls) for radioimmunoassay of insulin. Results. Insulin levels (pg/ml) are shown in the table (* = $p < 0.05$ vs control rats; Δ = $p < 0.05$ vs basal insulin level).

	Time				
	Basal	10	30	60	120
Control	258±75	389±78	238±23	280±72	292±67
GRP	242±60*	747±136*Δ	2204±480*Δ	295±76Δ	301±56Δ

Conclusions. These results indicate that GRP is a potent stimulant of insulin secretion.

MEASUREMENTS OF CHOLECYSTOKININ AND PANCREATIC IMMUNOREACTIVITY IN RATS: EFFECTS OF BOMBESIN. P. L. Rayford, K. Inoue, D. McKay, and P. Chowdhury. Dept. of Physiology-Biophysics, Univ. Arkansas Med. Sciences, Little Rock, AR 72205.

This study was conducted to determine if blood and tissues of rats contain immunoreactive cholecystokinin (CCK) and pancreatic polypeptide (PP). Methods: Groups of 6 rats were infused via the jugular vein with saline, 5 µg or 25 µg of bombesin (BBS). Blood samples were collected before and during BBS. Rats were sacrificed, the duodenum (for CCK) and pancreas (for PP) were removed and extracted for measurement of peptides by radioimmunoassay. Results: Slopes of dose-response curves for rat plasma and duodenal extracts were not siginificantly different from that of 99% pure CCK-33. In addition, graded doses of bovine PP and rat plasma generated parallel dose-response curves. Plasma levels of CCK and PP did not change with saline or with 5 µg of BBS. CCK levels increased from a control value of 59±16 pmol/ml to 89±17 and to 94±16 pmol/ml ($p<.05$) and PP increased from 7±2 to 14±2 and 16±2 pmol/ml ($p<.05$) with 25 µg BBS: integrated values for CCK and PP for the 30 min period were significantly greater than that found with saline. Conclusion: Tissues and blood of rats contain immunoreactive CCK and PP. Blood levels of both substances increased with 25, but not 5 µg of BBS suggesting that the response to BBS is dose related. Supported by NIH Grant AM30415-01.

MEMBRANES ENHANCE ALDOSTERONE BINDING IN RAT KIDNEY CYTOSOL PREPARATIONS. Martin Diesterhaft, Pavel Komanicky and John R. Higgins. Depts. of Internal Medicine and Biochemistry, Texas Tech University School of Medicine and the VAH Medical Center, Amarillo, Texas, 79106.

We tested rat kidney membranes for effects on the binding of ^{3}H-Aldosterone to receptor. Kidneys from adrenalectomized Sprague Dawley male rats were perfused with extraction buffer (50mM Tris phosphate, pH 7.5, containing 50mM Na_2MoO_4, 1mM EDTA, 10mM monothiolgylcerol and 10% glycerol), diced, dounced, and centrifuged at 105,000xg at 4°. The cytosols and membrane pellets were separated and the membrane pellets resuspended in the original extraction buffer. This suspension of membranes possessed little inherent binding activity. However, when aliquots of this membrane suspension were added to active cytosol preparations a maximal two to three fold stimulation of the original cytoplasmic binding activity was seen. This membrane associated stimulation of binding activity could not easily be extracted and was a proportional, saturable function of the amount of membranes added to the cytosol. Maximal activation required Na_2MoO_4 both in the isolated cytosol and membrane preparations. We conclude that factors tightly bound to cell membranes are capable of activating mineralocorticoid receptors.

DIHYDROALPRENOLOL BINDING TO ADIPOCYTES OF HYPERTENSIVE RATS. Karl M. Nelson and Judy A. Spitzer. Department of Physiology, LSU Medical Center, New Orleans, LA 70112.

The lipolytic response of adipocytes from spontaneously hypertensive (SH) rats to norepinephrine has been shown to be blunted. One means by which norepinephrine-stimulated lipolysis can be influenced is via modulation of hormone receptor number and/or affinity. In the present study, (-) [^{3}H] dihydroalprenolol (DHA) was used to assess B-adrenergic binding to intact fat cells from normotensive and SH rats. Nonspecific binding was defined as the amount of DHA bound in the presence of 1 mM isoproterenol. The amount of DHA specifically bound by adipocytes of normotensive rats was the same as that bound by fat cells from SH rats. The dissociation constant (Kd) and number of binding sites (Bmax) were not different in the two populations of adipocytes. Increasing amounts of adrenergic agonists and antagonists decreased total DHA binding to fat cells from normotensive rats. The displacement of DHA was significantly diminished with fat cells from SH rats (Supported by George S. Bel Award from American Heart Association of Louisiana, Inc.).

A NEW FLUORESCENCE SPECTROSCOPIC TECHNIQUE FOR MAPPING THE NUCLEOTIDE-BINDING SITE OF cAMP-DEPENDENT PROTEIN KINASE CATALYTIC SUBUNIT. D. Bhatnagar and R. Roskoski, Jr., Department of Biochemistry, LSU Medical Center, New Orleans, LA 70112. (SPON: J.R. Porter)

One approach to understanding the mechanism of protein kinase action is to examine the spatial restrictions of the site(s) involved in catalysis by studies including chemical modification and the use of analogs of natural ligands. Using the latter approach, we have developed a rapid, non-destructive fluorescence spectroscopic method for mapping the ATP-binding site in the catalytic subunit of cAMP-dependent protein kinase. This is achieved by displacing the fluorescent nucleotide lin-benzo-ADP with other nucleotides and analyzing the binding affinity of the displacing nucleotide for the catalytic subunit. The K_d's obtained for Adenosine, AMP, ADP, AMP.PNP of 32, 642, 10, and 53 μM, respectively, by fluorescence displacement agree very well with the K_i's of 35, 640, 9.5 and 60 μM, in that order, obtained from steady state kinetic ments. (Supported by USPHS Grant NS-15994 and a post-doctoral fellowship American Heart Association-LA).

NTESTINAL HISTAMINE H_2-RECEPTORS: AGONIST DISSOCIATION CONSTANTS (K_A).
.A. Barker (Spon: L. Wecker). Dept. of Pharmacology, LSUMC, New Orleans, LA
0119

H_2-receptors are located on some myenteric plexus interneurons in guinea pig ntestine. Activation of these receptors results in longitudinal muscle contraction (J.P.E.T. 221:69, 1982). In this study the intestinal H_2-receptors were urther characterized by determining the apparent K_A values for the H_2-selective gonists, dimaprit (D) and 4-methylhistamine (4MH) by the method of Furchgott dv. Drug Res. vol. 3, 1966). Similar studies were carried out on a standard $_2$-receptor preparation, the guinea pig right atrium. Tissue H_2-receptor concenration was reduced by treatment with L-643,441, a selective irreversible H_2-antaonist (Pendelton et al., Fed. Proc. 41:Abs 7507, 1982). Concentration-effect urves were generated before and after treatment with L-643,441. Apparent K_A alues were determined from the equation $[A']/[A] = 1/q + ((1-q)/qK_A)[A']$. The pparent K_A values ($\bar{x}$ and 95% confidence limits) for 4MH on ileal and atrial H_2-eceptors respectively were 50 (26-74)μM (N=6) and 84 (57-112)μM (N=3) and for D ere 56 (39-72)μM (N=6) and 104 (56-153)μM, (N=3) respectively. These results uggest that ileal and atrial H_2-receptors represent a homogeneous population. Supported by BRSG #SO-RI-5376)

PRODUCTION AND CHARACTERIZATION OF MONOCLONAL ANTIBODIES AGAINST RAT APOLIPOPROTEINS. Laurence Wong, William Gallaher, and Paul S. Roheim. Department of Physiology, Louisiana State University Medical Center, 1542 Tulane Avenue, New Orleans, Louisiana 70112.

Hybridomas were produced in mice by injections of apolipoproteins into mice followed by cell fusion with mouse myeloma cell lines. The hybridomas were cloned by limiting dilution. The clones were then expanded and ascites fluid produced. The antibodies were characterized for their light and heavy chains. All the antibodies produced reacted to all classes of lipoproteins, i.e. VLDL, LDL, and HDL. Immunoblotting followed by autoradiography indicates that the antibodies reacted to fast-migrating peptides. 2D isoelectricfocusing SDS-PAGE immunoblotting indicates that 3 antibodies react to peptides having a pI greater than 6 or less than 4; and one reacted to 2 peptides having pI's of 4.5 and 4.6 and MW of 12,000. We concluded that 3 antibodies are directed against apoC_I and one against apo C_{III}. (Supported by NIH Program Project #HL25596.)

PREPARATION OF VESICLES WITH TRANSPORT CAPABILITY FROM CULTURED FIBROBLASTS. Lewis C. Mokrasch, Department of Biochemistry, LSU Medical Center, New Orleans, La. 70119

As part of a study on membrane-bound transport enzymes in cultured fibroblasts from normal human donors and from muscular dystrophy victims, an active and stable model membrane system was needed. Vesicles have been useful in the study of transport related to other cell types, but nopreparation procedure was available for fibroblasts. Fibroblasts explanted from forearm skin biopsies were cultured using conventional technics. Cells in confluent cultures were complexed with a Ricinus lectin, swelled in hypotonic buffer, scraped free, homogenised, and centrifuged on a linear sucrose gradient. The membrane fraction was freed of lectin and concentrated by centrifugation. Vesicles were prepared by sonication of the membranes in isotonic buffers. Vesicles prepared in the presence of glutathione transported isotopic aminoacid. This transport plus radioiodination data indicated that the vesicles sealed with the correct sidedness. The presence of active ATPases suggest that the vesicles should be the fully competent model sought. The membrane and vesicle preparations were successful with several strains of fibroblasts.

DISTRIBUTION AND CATABOLISM OF Cd^{109} IN RATS. Parimal Chowdhury, Edward Harper, K. Inoue and Phillip L. Rayford. Dept. of Physiology-Biophysics, Univ. Ark. Med. Sci., Little Rock, AR 72205.

In rats, we have compared the effect of bolus and continuous injection of Cd on its distribution and catabolism. Methods: Fifteen rats were divided into 3 groups: Group I received saline, Group 2 received a bolus injection of 3.75 μCi Cd^{109}; Group 3 received 3.75 μCi Cd^{109} infusion for 50 min. Blood samples were collected at regular intervals for 120 min. The samples of lung, liver, kidney (R and L), heart, brain, muscle, fundus, antrum, ileum, cecum, colon, duodenum, pancreas and esophagus were collected and weighed individually. Cd^{109} in samples was measured with an auto-gamma counter. Percent distribution of Cd^{109} per gm of tissue was calculated. Results: Method of administration of Cd^{109} did not influence its distribution or catabolism. Blood levels of cadmium were 1.6±.06, .94±.06, .61±.20, .42±.25 and .35±.034 percent at 2, 4, 6, 8 and 10 min, respectively. Calculated T 1/2 was 7.4 min for the bolus injection and 8.4 min for the infusion. Maximum accumulation of Cd in liver was 4.54±.65, kidney 1.82±.09, and GI tract 3.92±0.025%. Neglible amounts were found in brain and muscle. Conclusion: The results suggest mechanisms for the catabolism of Cd^{109} are rapid and efficient which may be related to the large amounts of cadmium taken up by major target organ. (Supported by NIH Grant AM30415-01)

PRETERM RABBIT LUNG WASH DISATURATED PHOSPHATIDYLCHOLINE (DSPC) FOLLOWING HIGH FREQUENCY OSCILLATING (HFO) RESPIRATORY SUPPORT. Robert V. Kotas and Michael A. Moxley. The William and Natalie Warren Medical Institute, 6465 South Yale, Suite 1010, Tulsa, OK 74136-6783

We tested whether HFO (1,200 breaths/min) for one hour altered DSPC in lung wash of 30.5 day curarized (Pavulon) fetal rabbits removed by hysterotomy. Control animals consisted of littermates (LC) spontaneously breathing for one hour. The DSPC μmole/g lung dry weight (mean ± SEM) were not significantly different.

	Pellet	Supernatant
HFO	3.50 ± 0.51	1.38 ± 0.23
LC	3.02 ± 0.20	2.12 ± 0.22

Only washes free of blood were studied. Transcutaneous carbon dioxide partial pressures of HFO were in the normocarbic range with compressed air as the gas source for the respirator. Small frequent movement of alveolar walls does not seem to change curarized fetal rabbit lung wash DSPC.

REGULATION OF SUBSTRATE OXIDATION IN ISOLATED HEART MYOCYTES BY DL-β-HYDROXYBUTYRATE. V. Chen, G. Wagner and J.J. Spitzer. Dept. of Physiology, LSU Medical Center, New Orleans, LA 70112

The oxidation of exogenous DL-β-hydroxybutyrate (βHB) and its effect on the oxidation of lactate, octanoate or palmitate were studied using freshly isolated, Ca^{2+}-tolerant heart myocytes from rats. Raising βHB concentration in the medium resulted in progressive increase in its oxidation rate which plateaued at 2 mM βHB. The presence of βHB (5 mM) inhibited the oxidation of 7.5 mM lactate by 60%. Octanoate (2 mM) and palmitate (0.4 and 1 mM) oxidation were decreased by 17 and 35% in the presence of βHB. The presence of palmitate, lactate or octanoate had little if any inhibitory effect on βHB utilization. In isolated cells from chronically streptozotocin-diabetic rats, βHB oxidation was reduced by 25%, whereas palmitate oxidation remained unaltered. Despite the reduced oxidation rate, βHB still competed favorably with palmitate, whereas palmitate exerted no effect on βHB oxidation in these cells. Thus, our findings suggest a possible regulatory role of βHB in myocardial substrate oxidation. (Supported by Louisiana Heart Association, NIH grants HL 23157 and HL 07098).

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DIETARY CHOLESTEROL INDUCED CHANGES IN APO A-I, APO A-IV AND APO E LEVELS IN ATHEROSCLEROTIC AND HYPERTHYROID DOGS. [redacted] S. Bobek. [redacted]

Two dietary regimens are currently used to induce hypercholesterolemia and atherosclerosis in dogs. Regimen I consists of feeding the dog thyroprotein with propylthiouracil and feeding a saturated fat diet containing 1% cholesterol (chol); regimen II consists of feeding the animals a vegetable shortening diet containing 5% chol by weight. To compare the effect of the two regimens on apoprotein levels, dogs were divided into 2 groups and assigned to regimen I (grp I, n=5) or regimen II (grp II, n=4). A significant increase in chol, apo A-I and apo E levels was detected within 5 days of initiation of the diets in both grps, and by 5 weeks, chol levels averaged 887 ± 200 mg/dl (mean ± SEM) in grp I versus 693 ± 103 in grp II. At that point, apoprotein levels (expressed as % of their basal value) were: grp I versus grp II: apo A-I 700 ± 240 versus 1100 ± 400; apo E 350 ± 100 versus 2350 ± 510; apo A-IV 300 ± 40 versus 800 ± 70; apo A-I 40 ± 10 versus 150 ± 10. Thus, both diets resulted in a marked increase in apo A-I, apo E, and apo A-IV levels. Regimen I produced a marked decrease in apo A-I levels whereas regimen II had little effect. It remains to be determined whether the decreased apo A-I is a result of hypothyroidism or some other dietary ingredient. (Supported by NIH Program Project #HL25506.)

MYOCARDIAL PROTECTION DURING ENDOTOXEMIA. M. E. [redacted], [redacted] and B. H. Perlmutter. Dept. of Physiology-Biophysics, University of Arkansas for Medical Sciences, Little Rock, Arkansas [redacted].

Mongrel dogs were anesthetized with pentobarbital, paralyzed with [redacted] bromide, ventilated on room air, administered [redacted] mg/kg E. coli endotoxin, and treated with ibuprofen (25 mg/kg). After 4 hrs, animals were [redacted] heart, from which a microsomal fraction was isolated by [redacted] tion. Ca^{++}-Mg^{++} ATPase activity and calcium uptake were measured using $^{45}CaCl_2$. Microsomes from hearts of control animals were able to sequester calcium at a rate of [redacted] ATP at a rate of [redacted] hearts of animals receiving endotoxin only were able to [redacted] rate of [redacted] P_i/mg/min. Microsomes isolated from hearts of animals receiving endotoxin and ibuprofen were able to [redacted] lyzing ATP at a rate of [redacted] from control. [redacted] cardial [redacted] Association.

MICROCOMPUTER EXTRACTION OF ELECTROCARDIOGRAPHIC PARAMETERS AND VECTORCARDIOGRAMS FROM THE LABORATORY RAT. John A. Ward, Mario Gamboa, Dale Robinson and Mary Daniel Healy. Division of Natural Sciences, Incarnate Word College, 4301 Broadway, San Antonio, Texas 78209.

The recording and analysis of rat electrocardiograms presents problems of noise reduction, frequency response, and standardization of measurements more serious than those associated with human ECG's. To improve the speed and reliability of measurements, programs for digitization, graphical analysis, measurement of intervals, and calculation of vectorcardiograms were developed on the Apple II Plus microcomputer. Comparisons of recorded ECG's with theoretical ECG's derived from the vectorcardiogram indicated that the rat conformed closely, though not exactly, to the Einthoven hypothesis. Alternative algorithms for automated measurement of ECG intervals were evaluated.
(Supported by NIH Grant No. 5-S06-RR-08170-05).

THE EFFECT OF MAGNESIUM DEFICIENCY ON THE RAT ELECTROCARDIOGRAM. John A. Ward, Dale Robinson and Mario Gamboa. Division of Natural Sciences, Incarnate Word College, 4301 Broadway, San Antonio, Texas 78209.

The electrocardiogram is dependent upon currents carried mainly by Na^{+}, K^{+} and Ca^{++}. To investigate the effect of magnesium deficiency on the rat electrocardiogram (ECG), 12 male Sprague-Dawley rats were divided into experimental and control groups of 6 rats per group. The experimental and control diets contained 4.5 and 650 ppm of magnesium, respectively. The ECG was monitored from the six standard frontal plane leads for 5 weeks. There was a significant prolongation in the QT interval in lead AVR in the experimental group after two weeks on the diet. Prolongation of the QT interval indicates delayed repolarization of ventricular myocytes. Delayed repolarization could be due to an effect of Mg^{++} either on K^{+} or Ca^{++} channels or on the ionic pumps responsible for maintaining the gradients of these ions.
(Supported by NIH Grant No. 5-S06-RR-08170-05).

THE EFFECT OF MAGNESIUM DEPRIVATION ON THE CONCENTRATIONS OF IONS IN THE BLOOD OF THE RAT. Mary D. Healy and Yvonne King. Division of Natural Sciences, Incarnate Word College, 4301 Broadway, San Antonio, Texas 78209.

The concentrations of ions Mg^{++}, Ca^{++}, K^{+} and Na^{+} were studied in the blood of three groups of rats. Nine Sprague-Dawley rats, weighing 190-220 grams were divided into three groups, each group containing three rats. The dietary regime for each group was as follows: Group 1. Magnesium sufficient (650 ppm), Group 2. Magnesium deficient (4.5 ppm) and Group 3. Lab Chow. After the animals had been on the diet for two weeks, surgery was performed in order to insert a cannula into the right external jugular vein. After cannulation, the animals were housed one cage and the cannulas were flushed once daily with 5% sodium heparin to maintain potency. Two 1 ml blood samples were taken daily from each animal. The atomic absorption spectrophotometry was used to determine the concentrations of Mg^{++}, Ca^{++}, K^{+} and Na^{+} the blood samples. The results of this study suggest low concentrations of Mg^{++} and K^{+} in correlation with high concentrations of Ca^{++} and Na^{+}. However, confirmation of these results is dependent upon further investigation.
(Supported by NIH Grant No. 5-S06-RR-08170-05).

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    3. Newell A. Simon HA. Programs as theories of higher mental processes. In: Stacy RW, Waxman BD, eds. Computers in Biochemical Research. New York, Academic Press, Vol 2:p000, 1965.

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calorie	cal	millimeter	mm
centimeter	cm	milliosmole	mOsm
counts per minute	cpm	minute	min
cubic centimeter	cm^3	molal (concentration)	*m*
Curie	Ci	molar (concentration)	M
degree Celsius (Centigrade)	-°C	mole	spell out
degree Fahrenheit	°F	molecular weight	mol wt
diameter	diam	nanogram	ng

gram	g	nanometer	nm
hour	hr	normal (concentration)	*N*
inch	in	osmole	Osm
inside diameter	i.d.	ounce	oz
intramuscular	im	outside diameter	o.d.
intraperitoneal	ip	parts per million	ppm
intravenous	iv	percent	%
kilocalorie	kcal	picogram	pg
kilogram	kg	revolutions per minute	rpm
liter	spell out	second	sec
meter	m	specific activity	sp act
microliter	μl	square centimeter	cm^2
micrometer	μm	square meter	m^2
milligram	mg	subcutaneous	sc
milliliter	ml	volt	V
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